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Groping through the black box of variability: An integrative taxonomic and nomenclatural re-evaluation of *Zospeum isselianum* Pollonera, 1887 and allied species using new imaging technology (Nano-CT, SEM), conchological, histological and molecular data (Ellobioidea, Carychiidae)

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We especially wish to dedicate this work to the Slovenian malacologist, Jože Bole in commemoration of the 20th anniversary of his death (December 25, 1995). His immense contributions to malacology, especially in *Zospeum* research, have provided the knowledge base upon which we rely today.

Abstract

The minute troglobitic species, *Zospeum isselianum* Pollonera, 1887 (Eupulmonata: Ellobioidea, Carychiidae) is widely distributed within its Southern Alpine-Dinaric range. Its broad distribution and highly variable shell has caused this species to be historically lumped into its current taxonomic state of ambiguity. In an integrative taxonomic approach, phenotypic and genotypic data are synthesized to assess the intraspecific variability recently inferred for this taxon. We collected 16 *Zospeum* specimens in the Slovenian Alpine Arc encompassing the type locality for *Z. isselianum*. The material comprises five morphologically recognized (sub)species. The species are re-evaluated using SEM, X-ray nanotomography (nano-CT), conchological, histological and molecular data.

Four well-defined lineages are present, which can be attributed to i) *Z. isselianum* s.str. from its new type locality (Turjeva jama), ii) a highly morphologically variable lineage that so far cannot be attributed to a single morphospecies, iii) *Z. kupitzense* A. Stummer, 1984 (raised to species rank) and iv) a lineage comprising the two subspecies *Z. alpestre alpestre* (Freyer, 1855) and *Z. alpestre bolei* Slapnik, 1991 plus *Z. isselianum* individuals. The latter is treated as a single taxon *Z. alpestre*. After considering the severely degraded syntype material of *Zospeum isselianum*, we provide a taxonomic re-description and propose a neotype for this species. Furthermore, new diagnostic information is revealed regarding the columella of *Zospeum isselianum* and allied species. Detailed anatomical study reveals new structural aspects of *Zospeum* morphology and provides groundwork for future investigations.

Keywords

Neotypification, lumped species, subterranean microgastropods, Byne's degradation, species flock

Introduction

Systematic research is undergoing a major transition in the assortment of tools and terminology now available in today's taxonomic toolbox. In addition, species delimitation, the practice of recognizing and determining species boundaries, has expanded to evaluate the current inflation of species concepts generated to infer biological information (de Queiroz 2000, Katz et al. 2015). Speciation is an ongoing process influencing traits at varying temporal scales. In assessing subterranean biodiversity, familiar concepts generally used to describe speciation in metapopulations (i.e. monophyly, diagnostic morphology, distinct ecology, reproductive isolation etc.) are not directly practicable. Morphological stasis or overlapping intra- and interspecific character variability often confuse interpretation of species (hypotheses) and confound conventional species delimitation approaches (de Queiroz 2007, Katz et al. 2015). In our case with subterranean ellobioid microsnails, integrative taxonomy provides the most tangible answer to this problem since it pulls together data derived from different data sources including comparative morphology, genetics, ecology and geology to enable inferences about founded, but sketchy species hypotheses. In addition, new imaging technology in conjunction with molecular information opens new perspectives for interpretation, description and definition of morphology (Stoev et al. 2013).

In this work, we conduct the requisite transition from molecular species delimitation approaches (Weigand et al. 2011, 2013) to species clarification in a geographi-

cally compact group of taxonomically challenging troglobitic microgastropods. We test the efficacy of integrative taxonomy using novel imaging techniques to address the large degree of conchological variability in *Zospeum isselianum*, a taxonomic orphan of frequent synonymies and lumped species designations within the Carychiidae (Pollonera 1905, Stossich 1899, Absolon 1916, Kuščer 1932, Alzona 1971, Bole 1974, Giusti and Pezzoli 1982). Geographically proximal congeners to the type locality of *Z. isselianum* are assessed in a molecular and conchological context. This work augments the initial studies of Giusti (1975) and De Mattia (2003) and presents the first comprehensive anatomical investigation using a contempory framework for *Zospeum* Bourguignat, 1856. Since recent work on subterranean microgastropods using X-ray nanotomography (nano-CT) and scanning electron microscopy (SEM) effectively differentiated genera of small hypselostomatid snails (see Jochum and Malkowsky 2013, Jochum et al. 2014) and highlighted interspecific variation within shells of carychiid morphospecies (Jochum et al. 2013, 2015b), we apply the same imaging approach in this study.

Zospeum is known to inhabit caves of Northern Spain to the Balkan Dinarides. Most species were described in the latter half of the 19th and 20th Centuries based on shell characters such as whorl number, aperture dentition, and shell size as well as shape and number of lamellae circumscribing the columella (Kobelt 1901, Gittenberger 1973, 1980, Bole 1974, Maier 1982 unpublished data, Slapnik 1991). Twenty-four species and ten subspecies have been described (Bank 2013, Weigand 2013, Weigand et al. 2014, Jochum et al. 2015a). Ongoing research however, questions the validity of many of these species designations and simultaneously points to an even greater number of cryptic species. Contrary to the extensive work of Bole (1974), aspects of phenotypic variability and the availability of enough *Zospeum* material for comparison were seldom issues during earlier taxonomic eras. An additional impediment includes the fact, that many shells in historical collections were often culled from river debris and likely originated from populations inhabiting any number of caves located distances away from the specific river, town or cave system considered to be the official type locality.

Six anatomical studies provide the current knowledge upon which morphological findings of this investigation are based (Bole 1974, Giusti 1975, Maier 1982 unpublished data, De Mattia 2003, Martins 2007, Dörge 2010, unpublished data). Weigand et al. (2011) conducted the first molecular analysis (DNA barcoding) of worldwide Carychiidae, including seven Dinaric *Zospeum* morphospecies. In this work, high intraspecific variation was reported for *Zospeum* collected in different caves while a high incidence of cryptic allopatric speciation for several taxa was uncovered. A subsequent study evaluated original species assignments (Weigand et al. 2013). These authors not only found many morphologically unrecognized evolutionary lineages within this taxon, but also revealed that several alleged *Zospeum* species are ambiguously classified products of past taxonomic lumping traditions. However, in light of this past work, consideration of historical designations and recent investigations cannot ignore the fact that conventional molecular genetic methods, on the one hand, have not only solved

many taxonomic conundrums in biological systematics, but have, on the other, also confounded the situation by uncovering more complicated patterns of unrecognized genetic variability, which otherwise, may never have been detected (Sauer and Hausdorf 2012, Jörger and Schrödl 2013, Duda et al. 2014, for detailed discussion of cryptic speciation see Jörger and Schrödl 2013). Still, and in all due respect of this work, we strive for clarity and understanding of these microgastropods: for accuracy in biodiversity studies, for basic biogeographical investigations and for forming conservation strategies.

Past anatomical investigations have involved intrepid and remarkable dissections of some now dubious, *Zospeum* designations (Bole 1974, Giusti 1975, De Mattia 2003). Giusti (1975) conducted the first anatomical investigations on *Zospeum spelaeum* (Rossmässler, 1839) and *Zospeum tellinii* Pollonera, 1889. De Mattia (2003) specifically compared the genital anatomy of *Zospeum spelaeum* from six populations from different caves and one population of *Z. isselianum* (Grotta Nuova di Villanova) from the widely cavernous region of Trieste. These investigations revealed no substantial differences in morphology between and within different populations. Bole (1974) anatomically examined four different species and subspecies of *Zospeum*, emphasizing that his findings provided nothing taxonomically remarkable. Additional interpretation has remained limited due to the difficulties of collecting enough live material to compare different species. Other investigators such as Maier (1982, unpublished data) described *Z. spelaeum schmidtii* (Frauenfeld, 1854) using serial cross sections to reconstruct the anatomy. Dörge (2010, unpublished data) performed sketchy 3D reconstructions to depict the anatomy from stacked histological serial sections for a *Zospeum* species from Konečka zijalka (Kamnik-Savinja Alps, Slovenia). In regards to *Z. isselianum* here, we question the current value of these investigations for *Z. isselianum* species assignments. In light of these anatomical investigations and regarding our proposal to the International Commission on Zoological Nomenclature (ICZN Art. 81) for designating a neotype (Art. 75.5) for *Z. isselianum*, we present here the first perspective of specific organ structures for *Zospeum* using histology in conjunction with shell analysis of geographically proximal allied species and molecular data. For diagrammatic representation, consult Giusti (1975, figs 1, 2) and De Mattia (2003, figs 25, 26).

Zospeum isselianum has up to now, represented a Southern Alpine and Dinaric taxon (Pollonera 1887, 1905, Stossich 1899, Alzona 1971, Bole 1974, Giusti and Pezzoli 1982). It is known to inhabit caves from a few meters above sea level to an altitude of 2000 m (Hamann 1896, Zilch and Jaeckel 1962, Bole 1974, Pezzoli 1992, Slapnik 1991, 1994, Slapnik and Ozimec 2004). An excursion through the literature shows this species has been found in: 17 caves in Italy (Pollonera 1887, Alzona 1971, Maier 1982, unpublished data); five caves and one sediment sample in Austria (Maier and Bole 1975, Mildner 1976, Maier 1982, unpublished data); 169 caves, 16 springs and 37 sediment samples from river basins in Slovenia (Kuščer 1925, Bole 1960, 1974, Maier and Bole 1975, Slapnik 1991, 1994, 2001); 70 caves, five springs and one river basin sediment sample from Croatia (Brusina 1870, Langhoffer 1912, 1915a, 1915b, Slapnik and Ozimec 2004) and in seven caves in Bosnia and Herzegovina (Bole 1974). In light of our current reconsideration of earlier species designations and the requisite

Figure 1. *Zospeum isselianum* potential distribution collated from the literature and museum collections (I. Sajko, CSR SASA).

aspects of cryptic speciation in these enigmatic snails, this tallies to 312 potential, but highly questionable localities for *Zospeum isselianum* (Fig. 1). The localities included in the map (Fig. 1) are listed in Suppl. material 1.

Material and methods

Material is housed in the following collections:

CBSS	Croatian Biospeleological Society, Zagreb, Croatia
CSR SASA	Centre for Scientific Research of the Slovenian Academy of Sciences and Arts in Ljubljana, Slovenia
MSNG	Museo Civico di Storia Naturale »Giacomo Doria«, Genoa, Italy
MHNG	Museum d'Histoire Naturelle de Genève, Geneva, Switzerland
MZUT	Museo Regionale di Scienze Naturali Sezione di Zoologia, Torino, Italy
NHMW	Naturhistorisches Museum Wien, Vienna, Austria
NMBE	Naturhistorisches Museum der Burgergemeinde Bern, Bern, Switzerland
RS	Rajko Slapnik malacological collection, Kamnik, Slovenia
SMF	Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt am Main, Germany
SMNH	Slovenian Museum of Natural History Ljubljana, Slovenia

Taxon sampling

Material was collected from caves of the Julian Alps (Turjeva jama) and the Kamnik-Savinja Alps of Slovenia, which represent the southeastern-most extension of the Alpine topographic chain at the transition to the Dinarides (Fig. 1, Table 1). The two ranges cover about 3000 km² and are located in northwestern Slovenia on the border with Italy and Austria (Figs 1, 3). The highest peaks scale to 2800 m in elevation, producing between 500 and 2000 m of topographic relief.

Table 1. Overview of cave locality information. For each of the seven cave localities the investigated specimens, their museum ID, latitude and longitude data, date of collection, elevation and a measurement of the ambient cave temperature are given. Leg. Rajko Slapnik. Museum ID's refer to CSR SASA, RS, or SMNH collections.

Cave locality	Specimen ID (museum ID)	Lat.	Long.	Date	Elevation [m]	air T [°C]
Turjeva jama, Robič, Kobarid, Slovenia (region locus typicus)	1 (37013)	46.2435	13.5046	19.10.2007	253	9.3 (on 01.06.2013)
	2 (37013a)			19.10.2007		
	3 (RS0063a)			01.06.2013		
Ložekarjeva jama, Olševa mountain, Kamnik-Savinja Alps, Slovenia	4 (38474a) (MC SMNH 3291)	46.4268	14.6240	28.09.2009	1050	7.9 (on 28.09.2009)
	5 (38474a)			28.09.2009		
Konečka zijalka, Šmihel nad Mozirjem, Mozirje, Kamnik-Savinja Alps, Slovenia	6 (37698a) (21675)	46.4024	14.9393	16.08.2008	820	7.5 (on 16.08.2008)
	7 (37698a)			16.08.2008		
	8 (37698a)			16.08.2008		
Potočka zijalka, Olševa mountain, Kamnik-Savinja Alps, Slovenia	9 (40600a-1) (RS0059)	46.4493	14.6693	08.06.2012	1630	5.5 (on 08.06.2012)
	10 (40600a-2)			08.06.2012		
Jama na Zgornjih Brsnikih (= Jama pod Farjevim plazom, Jama pod Mokrico), Mokrica mountain, Kamniška Bistrica, Kamnik-Savinja Alps, Slovenia	11 (39948a) (MC SMNH 2216)	46.3093	14.5832	05.06.2001	980	6.0–10.2 (in 1997)
	12 (39948a)			05.06.2001		
	13 (39948a)			05.06.2001		
Tomažičeva zijalka, Podvolovljek, Kamnik-Savinja Alps, Slovenia (locus typicus)	14 (40596a-1)	46.3134	14.6982	30.04.2012	590	8.9 (on 30.04.2012)
	15 (40596a-2)			30.04.2012		
Kamniška jama, Zeleniške Špice, Kamniška Bistrica, Kamnik-Savinja Alps, Slovenia	16 (40595a-1)	46.3386	14.6124	01.05.2012	1400	5.1–6.1 (between 01.05.2003–01.05.2004)
Ihanščica cave, Ihan, Ljubljana, Slovenia	30014, 30042 (RS0103; RS0104)	46.1216	14.6476	17.5.1969 11.10.2013	415	Not recorded

Geologically, the Slovenian Alps belong to the Southern Alpine thrust-belt, which formed during the last 20 MY and is still tectonically active, as evidenced by considerable seismic activity (Fig. 4) (e.g. Vrabec and Fodor 2006). Karstification-prone, Mesozoic carbonate platform rocks blanket most parts of the range. Present-day vertical karst drainage favors the development of vadose shafts, the prevailing cave type. However, many remnants of phreatically formed horizontal cave systems are also present. These remnants of horizontal cave systems suggest rapid and major neotectonic uplift of the area.

Since historical species designations are geographically very general (i.e. "Carniola" or "Krain" for the Eastern Alpine region including southern Austria and Slovenia) and taxonomic data was recorded during eras of former political boundaries, geographical designations and taxonomic traditions, we have endeavored to collect live material of *Z. isselianum* and allied species as close to where the initial sites of species descriptions may have been. We remark however, that although the subspecies, *Zospeum alpestre likanum* Bole, 1960 could be considered in a broader scope of this investigation, it's geographic type locality (Gornja Cerovačka pećina, Gračac, Croatia) is located distant from the core, proximal Eastern Alpine sites comprising this study (Fig. 3). Our dataset consists of 16 *Zospeum* specimens (Table 2). These specimens either originate from previous studies (see Weigand et al. 2011, 2013) or were sampled within the scope of this study (Table 2).

Table 2. Overview of the morphological and genetic results. The 16 specimens (#) can be attributed to five morphospecies. The genetic delimitation reveals four clusters (color-coded), with four lineages (L1–L4). Individual BOLD-ID. (http://www.boldsystems.org/) of each specimen and a link to the broader study of Weigand et al. (2013) are given. New material of the present study is indicated by an asterisk (*).

Morphospecies	#	Lineage	BOLD-ID	Comments
***Z. isselianum* Pollonera, 1887**	1	L1	BARCA121-10	same as Z6 from Weigand et al. 2013
	2	L1	BARCA213-15*	same as Z6 from Weigand et al. 2013
	3	L1	BARCA214-15*	same as Z6 from Weigand et al. 2013
	11	L3	BARCA122-10	same as Z5 from Weigand et al. 2013
	12	L3	BARCA216-15*	same as Z5 from Weigand et al. 2013
	13	L3	BARCA217-15*	same as Z5 from Weigand et al. 2013
	6	L2	BARCA123-10	same as Z7 from Weigand et al. 2013
	7	L2	BARCA124-10	same as Z7 from Weigand et al. 2013
	8	L2	BARCA215-15*	same as Z7 from Weigand et al. 2013
Z.* cf. *amoenum	9	L2	BARCA211-13*	same as Z7 from Weigand et al. 2013
	10	L2	BARCA212-13*	same as Z7 from Weigand et al. 2013
Z. alpestre alpestre	16	L3	BARCA218-15*	same as Z5 from Weigand et al. 2013
Z. alpestre bolei	14	L3	BARCA219-15*	same as Z5 from Weigand et al. 2013
	15	L3	BARCA220-15*	same as Z5 from Weigand et al. 2013
Z. alpestre kupitzense	4	L4	BARCA125-10	same as Z1 from Weigand et al. 2013
	5	L4	BARCA126-10	same as Z1 from Weigand et al. 2013

Genetic delimitation

The barcoding region of the cytochrome c oxidase subunit I (COI) gene was amplified for 16 specimens from 7 caves including the morphospecies *Z. isselianum* (9 specimens, 3 caves), *Zospeum* cf. *amoenum* (Frauenfeld, 1856) (2 specimens, 1 cave), *Z. alpestre bolei* (2 specimens, 1 cave), *Z. alpestre alpestre* (1 specimen, 1 cave) and *Z. alpestre kupitzense* (2 specimens, 1 cave) (Table 2).

COI sequences for genetic delimitation were either retrieved from the BOLD project "BARCA" or generated anew within this study (Tab. 2). PCR and sequencing were performed following the protocols given in Weigand et al. (2011) without modifications. The alignment was created using the MAFFT-plugin in Geneious 5.4.7. The G-INS-I option for global homology and less than 200 sequences were used. Removal of the primer sequences for LCO1490 and HCO2198 was achieved by manual 3'- and 5'-trimming. The final alignment had a length of 655 bps. No gaps were present.

The Automatic Barcode Gap Discovery (ABGD) procedure was conducted (Puillandre et al., 2011) in order to semi-automatically detect a barcoding gap, which separates intra- and interspecific genetic diversity. The ABGD web server was used (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). We tested several combinations of relative gap width (X, from 0.2–2.0), the minimal prior intraspecific divergence (Pmin, from 0.001–0.05) and maximal prior intraspecific divergence (Pmax, 0.03–0.10). The number of bins (Nbins) was 20 and we used a Kimura (K80) TS/TV-ratio of 2.0. Additionally, specimens have been grouped and delineated by the reversed Statistical Parsimony (SP) approach after Hart and Sunday (2007). The approach was performed in TCS 1.21. Clusters were delineated with a connection limit of 99% (statistical probability).

Conchological assessment

To differentiate and compare the geographically proximal species comprising our study, available technology such as X-ray-nanotomography (nano-CT) and SEM are used to access new information from traditional morphological characters (external morphology, radula and sinuosity of the columellar lamella). The position and the degree of sinuosity of the columellar lamella is a widely accepted diagnostic character within the Carychiidae (Strauch 1977, Maier 1982, unpublished data, Bank and Gittenberger 1985, Medaković et al. 1999, Jochum et al. 2015). Bole (1974) and Slapnik (1991) on the other hand, found the parietal lamella and dentition in the aperture taxonomically more significant than the configuration of the columella. Focus here is directed from the inside out without the usual, perforating of windows in the shell and the risk of destruction of valuable material. In this work, X-ray perspectives enable us to reconsider characters for *Zospeum* such as the form of the columella (i.e. cylindrical, clavate, presence/absence of secondary basal dilatation, degree of twisting) as well as the relationship of the columellar lamella to the columella (i.e. degree of inclination,

extended or absent). In addition, SEM captures the superficial structural and textural aspects of the thin *Zospeum* shells.

Shells of three populations of *Zospeum isselianum* from three distinct genetic clusters (see results; L1, L2 and L3) were measured according to Slapnik (1991). This data is presented in Tables 3–4.

In order to assess historical species hypotheses in context here, access to type material housed in museum collections is paramount. Type material whenever possible has been obtained for this study (Figs 2A, G–P, 11A–E).

Histology and light microscopy

The formaldehyde-preserved specimens of conchologically determined (RS) *Zospeum isselianum* (Konečka zijalka) and *Z. isselianum* (Turjeva jama, neotype locality) were dissolved in a solution of 5% acetic acid and 10% formaldehyde to dissolve the shell. The snails were subsequently dehydrated in an increasing ethanol series. Pre-infiltration followed with a 96% ethanol and base liquid solution of Technovit 7100 (50:50 mixture) for two hours and then infiltrated over night following the manufacturer's instructions (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim/Ts., Germany). The specimens were subsequently embedded in cold-polymerizing hydroxyethyl methacrylate resin. Serial sections (1 µm) were prepared using the Leica RM2165 automatic Rotation Microtome (Leica Microsystems Nussloch, Germany), stained with toluidine blue and examined with a high-powered microscope (Leica DM LB2). Histological photomicrographs were taken using a digital camera (Leica DC 300F) and further processed using the software Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA).

Scanning Electron Microscopy (SEM)

The radula of *Zospeum isselianum* (Turjeva jama, neotype locality) was prepared according to Holznagel (1998), preserved in 96% ethanol and mounted onto a prepared SEM stub. The radula was sputtered with gold (1-2 x for 60 seconds) in the Agar Sputter Coater (Agar Scientific, Stansted, UK) and viewed in the high vacuum mode of the Hitachi S-4500 Scanning Electron Microscope (15 kV, probe current 20 -100 pA) using the secondary electron detector. Photographs were taken with DISS–Digital Image Scanning System 5 (Point Electronic, Halle, Germany).

Shells or preserved full-bodied snails selected for SEM were microscopically evaluated for stability (presence of cracks, shell thickness, evidence of erosion). The sturdiest shells were first wetted in a dish of 80% ethanol or water and then manually brushed clean of cave encrustations using fine, tapered dental brushes, whereby each specimen was gently rotated back and forth between the brushes until it was sediment free. Specimens were then mounted onto double-sided carbon tabs. The samples were sputtered and processed using the same systems as for the radula.

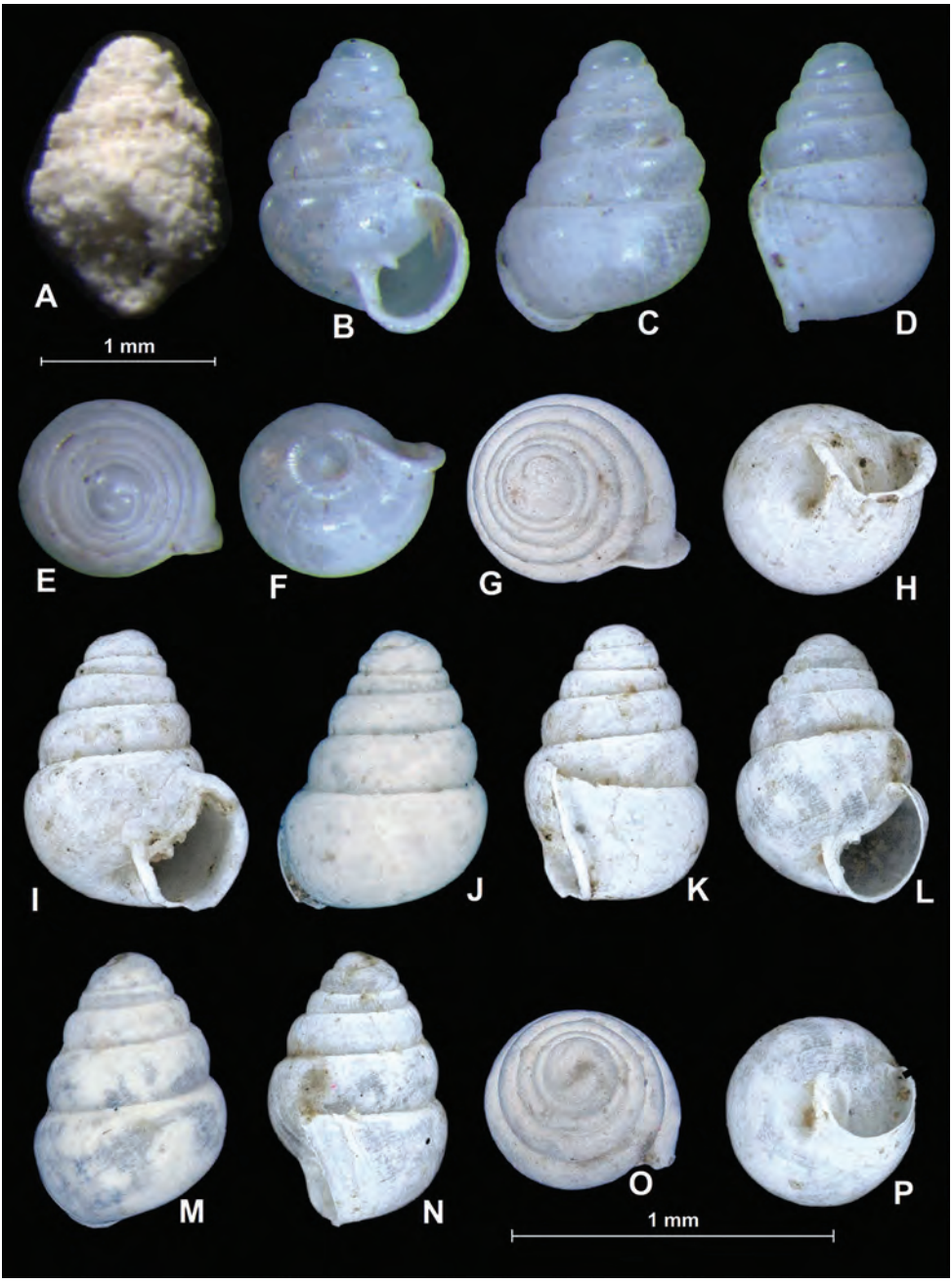

Figure 2. Historical material: **A** *Zospeum isselianum* syntype (MZUT M3232): upper mountains of Natisone River valley **B–F** *Zospeum isselianum* topotype: Turjeva jama **G–P** Specimens labelled as "Zospeum alpestre" in the Bourguignat Collection (MHNG 7898/2) from "Cav[erne]. de Carniole".

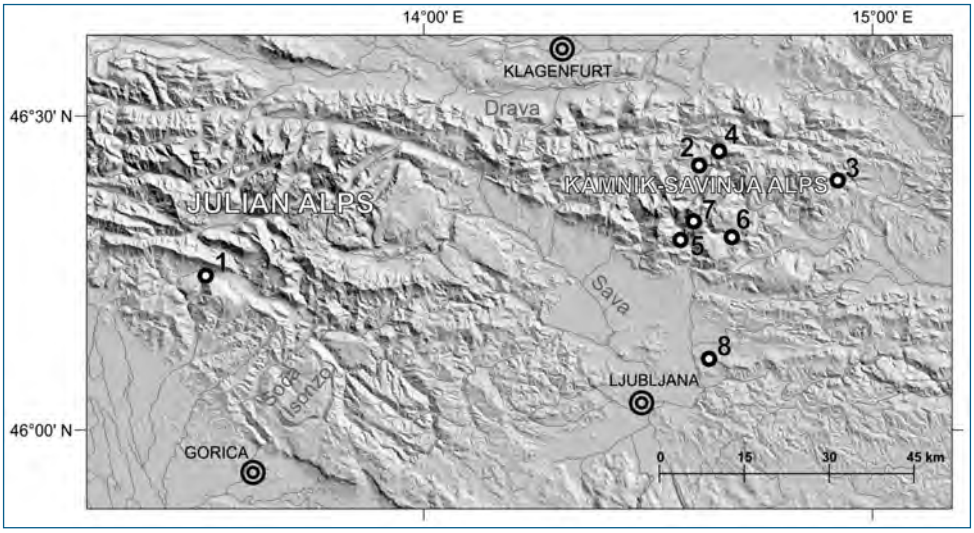

Figure 3. Geographical position and river drainage systems of the Julian Alps and the Kamnik Savinja Alps range. Sampling sites: **1** Turjeva jama **2** Ložekarjeva jama **3** Konečka zijalka **4** Potočka zijalka **5** Jama na Zgornjih Brsnikih **6** Tomažičeva zijalka **7** Kamniška jama **8** Ihanščica cave. Digital terrain model from Jarvis et al., 2008. River network data courtesy of Public Information of Slovenia, the Surveying and Mapping Authority of the Republic of Slovenia, DPK1000V (2008).

X-ray nanotomography

Specimens were imaged using a nano-computed tomography system (nano-CT), manufactured and developed by Bruker-Micro-CT/SkyScan (SkyScan 2011, Kontich, Belgium) at the Department of Experimental Radiology, Justus-Liebig University Biomedical Research Center Seltersberg (BFS), Giessen, Germany. The system contains an open pumped type X-ray source, a LaB6 cathode and a transmission anode consisting of a tungsten-coated beryllium window. Enhanced edge sharpness and submicron resolution are gained by a high-focused X-ray spot of <400 nm side length (see Langheinrich et al. (2010) for more details). Specimens of *Zospeum isselianum* (Turjeva jama, neotype locality) were mounted on a specimen holder and then fixed on a computer-controlled stage. They were then scanned 185° around their vertical axis in rotation steps of 0.23° at 80 kV tube voltage and 120 µA tube current. Reconstruction of cross sectional images was performed using a Feldkamp cone-beam reconstruction algorithm. Image resolution of the cross sectional images was 1,75 µm isotropic voxel side length with a grey scale resolution of 8 bit. Digital image post processing and visualization (maximum intensity projection MIP, volume compositing and summed voxel projection) were displayed using the ANALYZE software package (ANALYZE 11.0, Mayo Clinic, Rochester, MN, USA).

Digital imaging

Zospeum species were photographed using a Kontron-Electronik-ProgRes-3012 microscope camera (Jena, Germany) and a Leitz MZ12 stereomicroscope.

Results

Genetic delimitation

The ABGD approach delineates four clusters (Fig. 4): *Z. isselianum* (L1), *Zospeum* sp. (L2), *Z. alpestre* (L3) and *Z. kupitzense* (L4). The SP approach delineates five clusters, thereby splitting up L2 into two clades comprising *Z. isselianum* and *Z.* cf. *amoenum* specimens only, respectively. The maximal K2P-genetic distance between the two lineages in L2 is 2.3% (Table 3, Figs 5, 6), which is still within the threshold of intraspecific variability and thus, below the barcoding gap of 3.2% calculated for Carychiidae (Weigand et al. 2013). We refer to a more conservative approach here and consider four clusters (i.e. Lineage 1, L2, L3, and L4) whereby, the minimal K2P-interspecific distance in this study is 5.4% (Fig. 7).

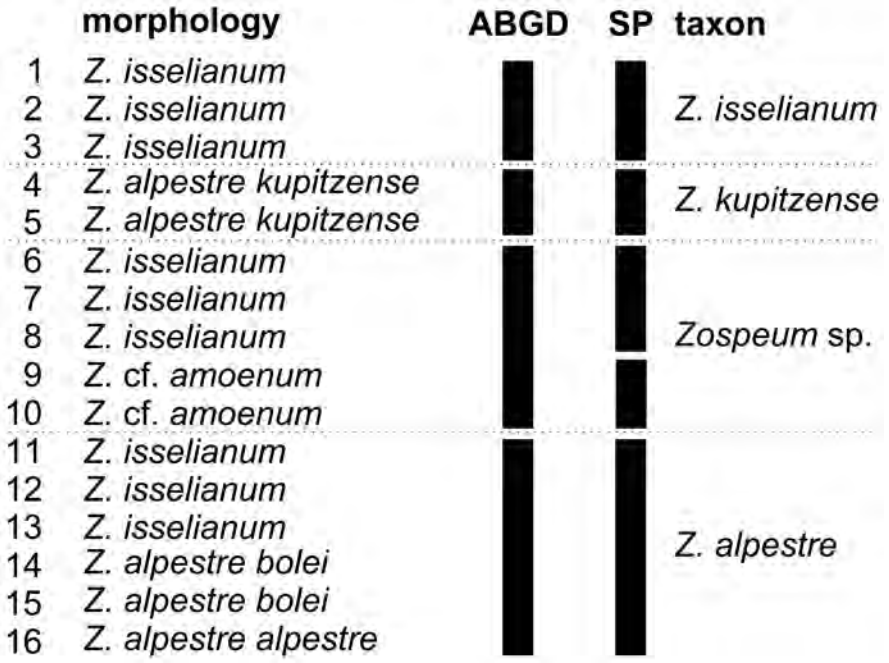

Figure 4. Overview of morphological assignments, molecular species delimitation results and integrative taxonomic treatment of each of the four clusters, which are indicated by the longitudinal black bars for either the ABDG or the SP approach. Numbers indicate individual specimens; ABGD = Automatic Barcode Gap Discovery; SP = Statistical Parsimony.

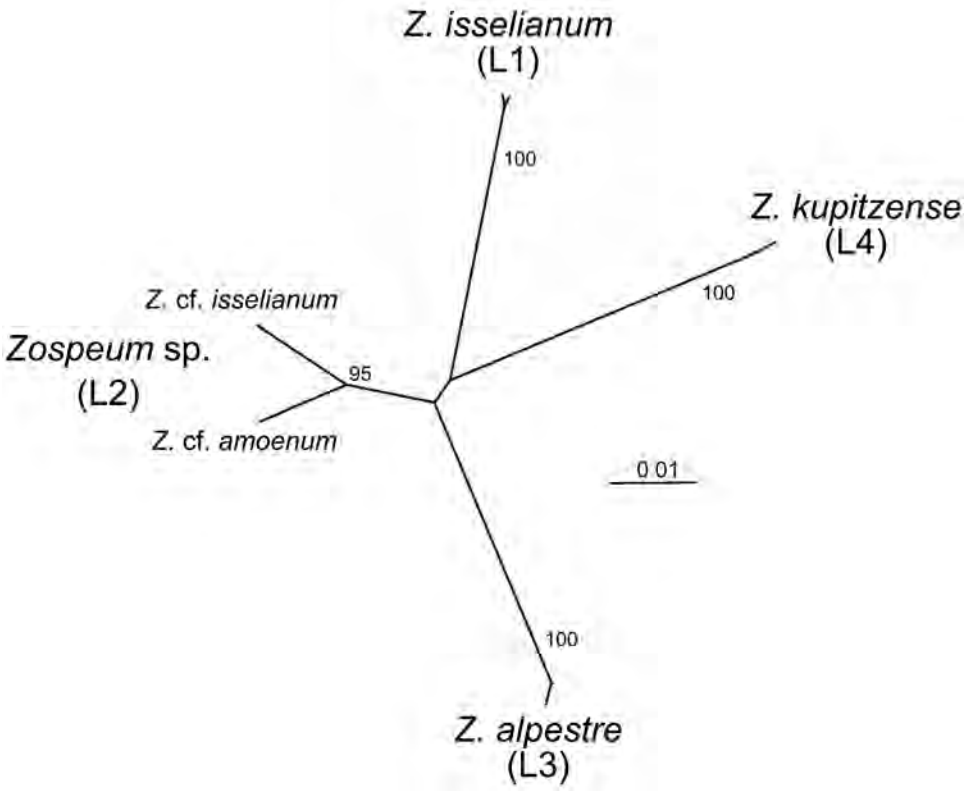

Figure 5. Network analysis for the molecular distinctness of the four delineated *Zospeum* species.

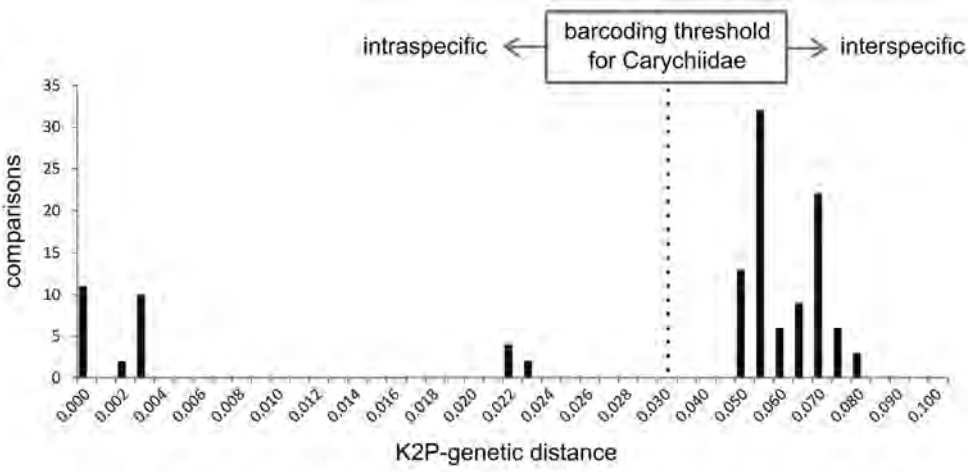

Figure 6. Overview of pairwise genetic distance measurements.

	morphospecies	genetic distance table														
		Z. isselianum														
1	***Z. isselianum***	**0.0**														
2	***Z. isselianum***	**0.0**	**0.0**													
3	***Z. isselianum***	**0.2**	**0.3**	**0.0**	*Z. kupitzense*											
4	*Z. alpestre kupitzense*	7.2	7.3	7.1	**0.0**											
5	*Z. alpestre kupitzense*	7.2	7.2	7.0	**0.2**	**0.0**	*Zospeum* sp.									
6	***Z. isselianum***	5.5	5.5	5.7	6.2	6.2	**0.0**									
7	***Z. isselianum***	5.4	5.4	5.6	6.2	6.2	**0.0**	**0.0**								
8	***Z. isselianum***	5.4	5.7	5.7	6.2	6.2	**0.0**	**0.0**	**0.0**							
9	***Z. cf. amoenum***	5.4	5.4	5.4	7.0	6.8	**2.3**	**2.2**	**2.2**	**0.0**						
10	***Z. cf. amoenum***	5.4	5.4	5.4	7.0	6.8	**2.3**	**2.2**	**2.2**	**0.0**	**0.0**	*Z. alpestre*				
11	*Z. isselianum*	7.0	7.0	7.2	7.6	7.4	5.7	5.6	5.6	5.6	5.6	**0.0**				
12	*Z. isselianum*	7.0	7.1	7.1	7.9	7.4	5.8	5.8	5.7	5.4	5.4	**0.0**	**0.0**			
13	*Z. isselianum*	7.0	7.1	7.1	7.9	7.4	5.8	5.8	5.7	5.4	5.4	**0.0**	**0.0**	**0.0**		
14	*Z. alpestre bolei*	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	**0.3**	**0.3**	**0.3**	**0.0**	
15	*Z. alpestre bolei*	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**
16	*Z. alpestre alpestre*	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**	**0.0**
specimen		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 7. Kimura–2-parameter (K2P) genetic distance table in %. Delineated species are marked with boxes. For each individual specimen, its morphospecies designation and genetic distance data to all remaining specimens are given.

Based on the four revealed evolutionary lineages, evidence for delineation using non-molecular methods encompasses four taxonomic consequences:

1) *Z. isselianum* Pollonera, 1887 (L1) is defined from the area of its new precise type locality (Turjeva jama, Robič, Kobarid, Slovenia) whereby a neotype is designated and a taxonomic re-description is provided.
2) We recognize a highly morphologically variable *Zospeum* sp. (L2) (Konečka zijalka) with conchological affinity to *Z. isselianum* and *Z.* cf. *amoenum*. However, *Z. amoenum* topotypic material is necessary before species status can be clarified. Histological examination of L2 provides novel insights into the anatomy of *Zospeum* in a comparative framework with other Ellobioidea.
3) *Z. alpestre* (L3) encompasses subspecies comprising the *Z. alpestre* complex, including *Z. alpestre alpestre* and *Zospeum alpestre bolei* plus three specimens of *Z. isselianum*.
4) *Z. kupitzense* (L4) is elevated from subspecies rank (*Z. alpestre kupitzense*) based on a clear differentiation of columellar configuration and lamellar extension outside the aperture.

Consequence 1: *Zospeum isselianum* (L1) neotype designation including shell morphology and genetics

Taxonomy

Family Carychiidae Jeffreys, 1830
Genus *Zospeum* Bourguignat, 1856

Zospeum isselianum Pollonera, 1887
Figures 2B–F, 8A–F, 9A–F

Zospeum alpestre — Bourguignat 1856 [non *Carychium alpestre* Freyer, 1855]
Zospeum isselianum Pollonera, 1887
Zospeum alpestre — Stossich 1899 [non *Carychium alpestre* Freyer, 1855]
Zospeum alpestre — Giusti and Pezzoli 1982 [non *Carychium alpestre* Freyer, 1855]

Data regarding the original type material. The two syntypes of *Zospeum isselianum* were collected in debris of the Natisone River in the northeastern Italian region of Friuli (Pollonera 1887). Pollonera remarked that A. Tellini collected the debris far north of the town of Cividale and that the specimens could only have originated from the upper, mountainous part of the Natisone valley, namely, the right tributary of the Isonzo River. The Natisone (Nadiža) flows from the Slovenian side of the Julian Prealps and courses the Slovenian-Italian border crossing into Eastern Friuli. Currently, the only known type material consists of a single shell (MZUT M3232) labeled "cotypus" from the Pollonera collection housed in the Museo Regionale di Scienze Naturali Sezione di Zoologia, Torino, Italy (Fig. 2A). Since this specimen (MZUT M3232) is unrecognizable due to Bynesian decay (also called "Bynes disease"), a granular efflorescence of the shell due to a physicochemical reaction involving acetate/formate compound salts and water, which largely erodes the shells (Callomon and Rosenberg 2012, Cavallari et al. 2014), it is no longer taxonomically informative. Moreover, since it cannot maintain nomenclatural stability (ICZN 2014, Art. 75.5), we propose the designation of a neotype (CSR SASA 37013) from the assumed type locality. Material of *Z. isselianum* selected for the neotype here was collected in Turjeva jama, located on the right bank of the Natisone (Nadiža) River by the village of Robič in the municipality of Kobarid, Slovenia. Geographically, this cave is most likely the source of the two shells initially presented to Arturo Issel and later dedicated to him in 1887 by Pollonera.

Original differential diagnosis (Pollonera 1887). "This species just resembles *Z. obesum* Schmidt, *Z. alpestre* Freyer (pars), and *Z. nyctozoilum* Bourg. From the first it differs because it lacks an obsolete columellar fold, because it has the umbilicus more open, the peristome less swollen, and the apex not acute. From the second it differs because it has the umbilicus less cramped, the apex less acute (indeed mammillatus), the sutures deeper and it has the last lap with a lower development. From the third it differs because the right margin is a little arched (not reflected) and it has the umbilicus more open (Issel)" (Pollonera 1887, translated from Italian by Massimo Prodan and Floriana Umani 2014).

Material examined. Neotype (CSR SASA 37013/37013a) (Fig. 8A–E). Conspecifics (BOLD-ID: BARCA121-10, ACCESSION NR: HQ171594) (Weigand et al. 2011)), SMF 341636 (Fig. 2B–F), NMBE 532009 (Fig. 8F): Turjeva jama, Robič, Kobarid, Julian Alps, Slovenia, 46.2435°N, 13.5046°E, alt. ca. 253 m, air temp. in cave 9.3° C, June 1, 2013. Leg. Rajko Slapnik.

Diagnosis. Shell minute, transparent when fresh, conical with entire, half-roundish and more or less thickened peristome; parietal lamella; columella non-introrse, secondary columellar dilatation just above umbilical indentation; columellar lamella inclinate.

Description. Measurements of neotype specimen CSR SASA 37013 (Fig. 8A–E): shell height 1.56 mm, shell diameter 1.07 mm, peristome width 0.75 mm, peristome height 0.84 mm.

Measurements of conspecifics from Turjeva jama are provided in Table 3 (based on neotype and conspecifics of neotype). Shells minute (Table 5), variable in height (1.05–1.63 mm), conical with about 5 whorls, regularly coiled, suture deep, whorls convex, more or less shouldered; aperture semicircular, peristome closely adhering to spire (Fig. 2D), thickened, higher than wide, taking up to ca. 35% of shell height; umbilicus closed, shallow depression; contact between the columellar edge of peristome and last whorl moderately long (0.34–0.59 mm); non-expressed parietal lamella in aperture continues only in traces within shell; parietal plica present or absent (Fig. 8F); penultimate whorl "sinks" into ultimate whorl at upper junction of peristome and penultimate whorl in profile perspective (Fig. 2D); columella twisted but non-introrse, slender, secondary columellar dilatation at base; columellar lamella inclinate, extended (Fig. 8A–D); secondary columellar dilatation equal to ½ distance of maximal upper columellar lamella extension; protoconch with pattern of spiral interconnected pits separated by zones of non-pitted bands (Fig. 9D–E); teleoconch with tightly spaced irregular spiral striae of densely interconnected pits (Fig. 9B, C, F); broader spiral bands interrupt finer rows of pitted striae (Fig. 9B).

Differential diagnosis (Figs 10, 11). Differs greatest from congeners here in columellar apparatus and surface microstructure; differs from *Zospeum* sp. (Konečka zijalka) by the more slender columella, secondary columellar dilatation equal to ½ distance of maximal upper columellar lamella extension, columellar lamella inclinate, extended; *Z.* cf. *amoenum* (Potočka zijalka) lacks columellar lamella, columella straight cylindrical (Fig. 10J); from *Z. kupitzense* by the planar less inclinate orientation of columellar lamella (Fig. 10A–F), less pronounced parietal lamella than in *Z. kupitzense* (Ložekarjeva zijalka) (Fig. 10E); from *Z. alpestre* (Kamniška jama) by the more slender columella, secondary columellar dilatation at base, columellar base of *Z. alpestre* clavate inflated, moderately attenuate (Fig. 10G); irregular striation of tightly interconnected pits of upper teleoconch differs from regular impressed wavy bands on teleoconch of *Z. kupitzense* (Fig. 9I–L); differs from rather regularly spaced rows of interconnected pits on teleoconch of *Z. amoenum* (Ihanščica cave). The differential diagnosis of genetic data is presented with the respective BOLD-ID numbers in Table 2.

Distribution. Although we question past distribution records (Fig. 1, Table 1), *Z. isselianum* likely occurs within a narrow radius encompassing the Southeastern Alpine

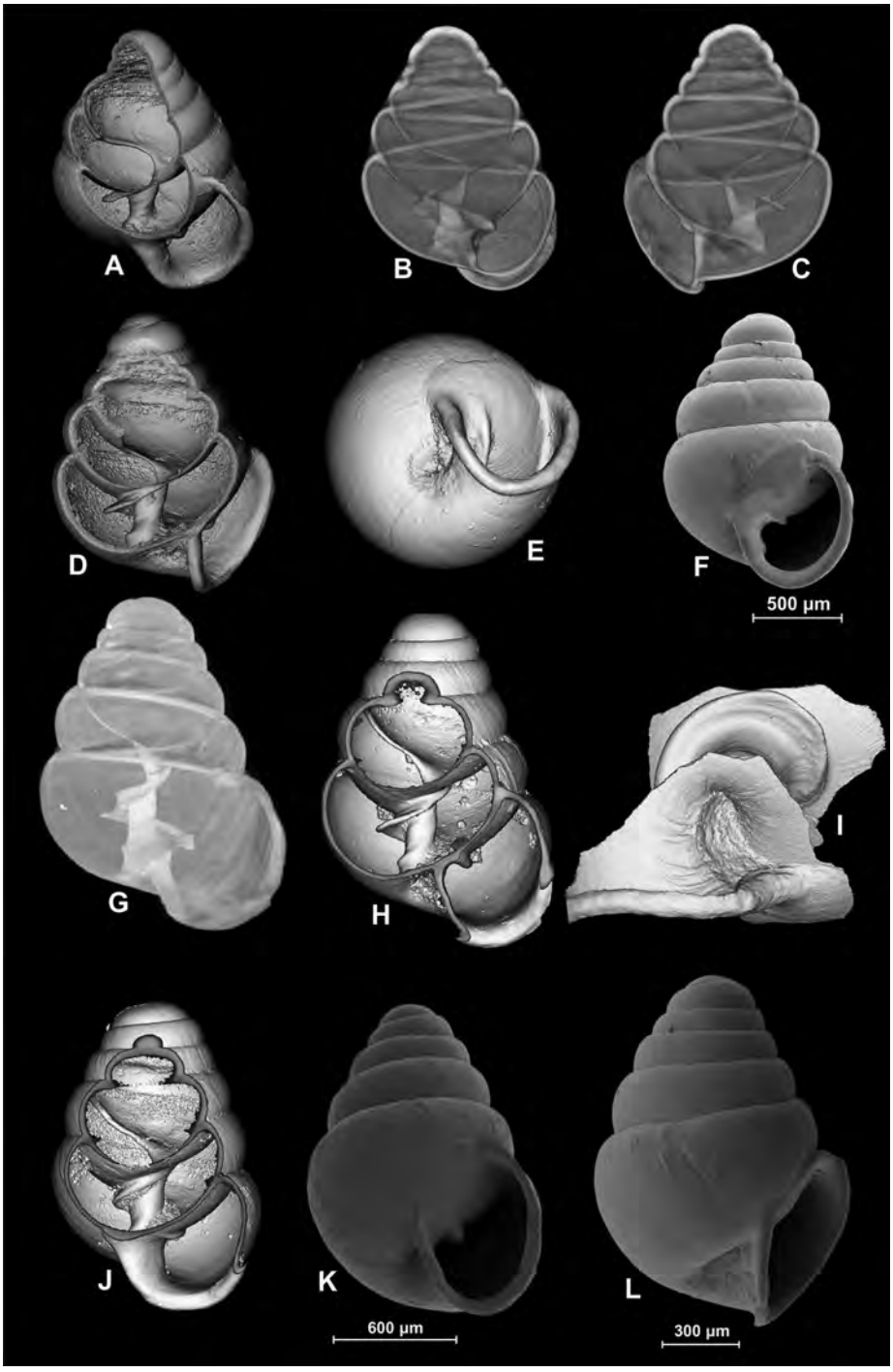

Figure 8. NanoCT–SEM Plate 2. **A–F** *Zospeum isselianum*, neotype (CSR SASA 37013), Turjeva jama **G–H** *Zospeum isselianum* (SMNH 2216), Jama na Zgornjih Brsnikih **I–L** *Zospeum* sp. (CSR SASA 37698a), Konečka zijalka.

Table 3. *Zospeum isselianum* conspecifics from neotype locality (Turjeva jama), CSR SASA 37013/01–10, Turjeva jama, Robič, Kobarid, Slovenia. **SH** Shell height. **SW** Shell width. **PH** Peristome height. **PD** Peristome diameter. **Lcol** Contact between the columellar edge of peristome and last whorl, all in mm.

	SH	SW	PH	PD	Lcol	SW/SH	AW/AH	Lcol/SH
37013/01	1.05	0.91	0.70	0.47	0.34	0.865	0.676	0.321
37013/02	1.35	1.02	0.71	0.58	0.46	0.755	0.813	0.341
37013/03	1.44	0.96	0.76	0.62	0.49	0.670	0.811	0.344
37013/04	1.36	0.95	0.69	0.53	0.54	0.698	0.766	0.396
37013/05	1.30	0.97	0.77	0.60	0.58	0.744	0.775	0.444
37013/06	1.39	0.95	0.70	0.58	0.52	0.686	0.825	0.377
37013/07	1.36	0.98	0.68	0.57	0.44	0.721	0.842	0.323
37013/08	1.37	0.92	0.68	0.60	0.54	0.673	0.893	0.396
37013/09	1.41	1.02	0.72	0.61	0.53	0.726	0.847	0.375
37013/10	1.31	0.96	0.68	0.56	0.55	0.737	0.813	0.423

Table 4. Shell dimensions. Mean, maximum (max), minimum (min), and standard deviation (sd) of shell measurements of 3 populations of *Zospeum isselianum*, Turjeva jama, N=40; Jama na Zgornjih Brsnikih N=40 and *Zospeum* sp. Konečka zijalka, N=32. **SH** Shell height. **SW** Shell width. **PH** Peristome height. **PD** Peristome diameter. **Lcol** Contact between columellar edge of peristome and last whorl, all in mm.

	SH	SW	PH	PD	Lcol	SW/SH	PH/PD	Lcol/SH
Zospeum isselianum Turjeva jama								
mean	1.35	0.97	0.70	0.58	0.49	0.72	0.83	0.36
max	1.63	1.09	0.84	0.62	0.59	0.86	0.92	0.45
min	1.05	0.91	0.65	0.47	0.34	0.63	0.68	0.24
sd	0.086	0.036	0.033	0.028	0.052	0.039	0.046	0.038
Zospeum isselianum Jama na Zgornjih Brsnikih								
mean	1.53	1.07	0.75	0.60	0.41	0.70	0.80	0.27
max	1.63	1.12	0.93	0.87	0.58	0.76	0.93	0.39
min	1.38	0.87	0.67	0.54	0.29	0.55	0.68	0.18
sd	0.051	0.055	0.044	0.053	0.060	0.037	0.048	0.0417
Zospeum isselianum Konečka zijalka								
mean	1.37	1.02	0.69	0.61	0.53	0.75	0.87	0.35
max	1.60	1.25	0.80	0.74	0.67	0.84	1.06	0.40
min	1.19	0.93	0.58	0.51	0.42	0.68	0.76	0.30
sd	0.084	0.066	0.051	0.039	0.066	0.037	0.055	0.053

region of contiguous cave and river systems of northeastern Italy, southern Austria, Slovenia and northwest Croatia (Bole 1974, Maier and Bole 1975, Mildner 1976, Slapnik 1991, 1994, Slapnik and Ozimec 2004).

Ecology. Live *Z. isselianum* were found in Turjeva jama on mud and muddy walls at the end of the cave and adjacent to the central pit located in the middle of the cave. Bats were seen in the vicinity of the collection site.

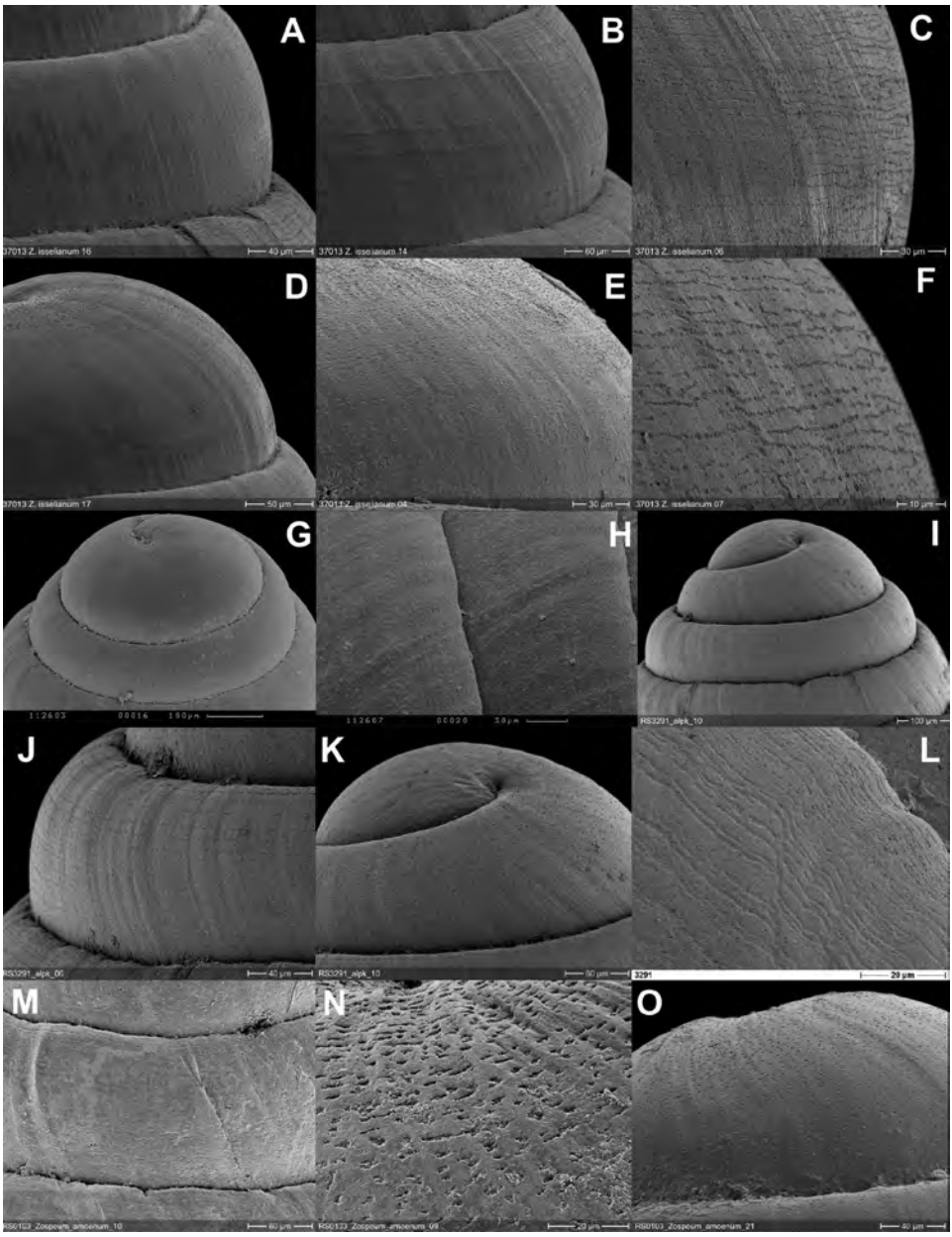

Figure 9. SEM Microstructures Plate 3. **A–F** *Zospeum isselianum* (CSR SASA 37013 conspecific), Turjeva jama **G–H** *Zospeum* sp. (CSR SASA 21675), Konečka zijalka **I–L** *Zospeum kupitzense* (SMNH 3291), Ložekarjeva jama **M–O** *Zospeum amoenum* (RS103), Ihanščica cave.

Conservation. In the caves sampled for this study, empty shells were sparsely found in sediment and live individuals sparsely populated certain cave walls during the summer season. These findings suggest that *Z. isselianum* occurred there for more than

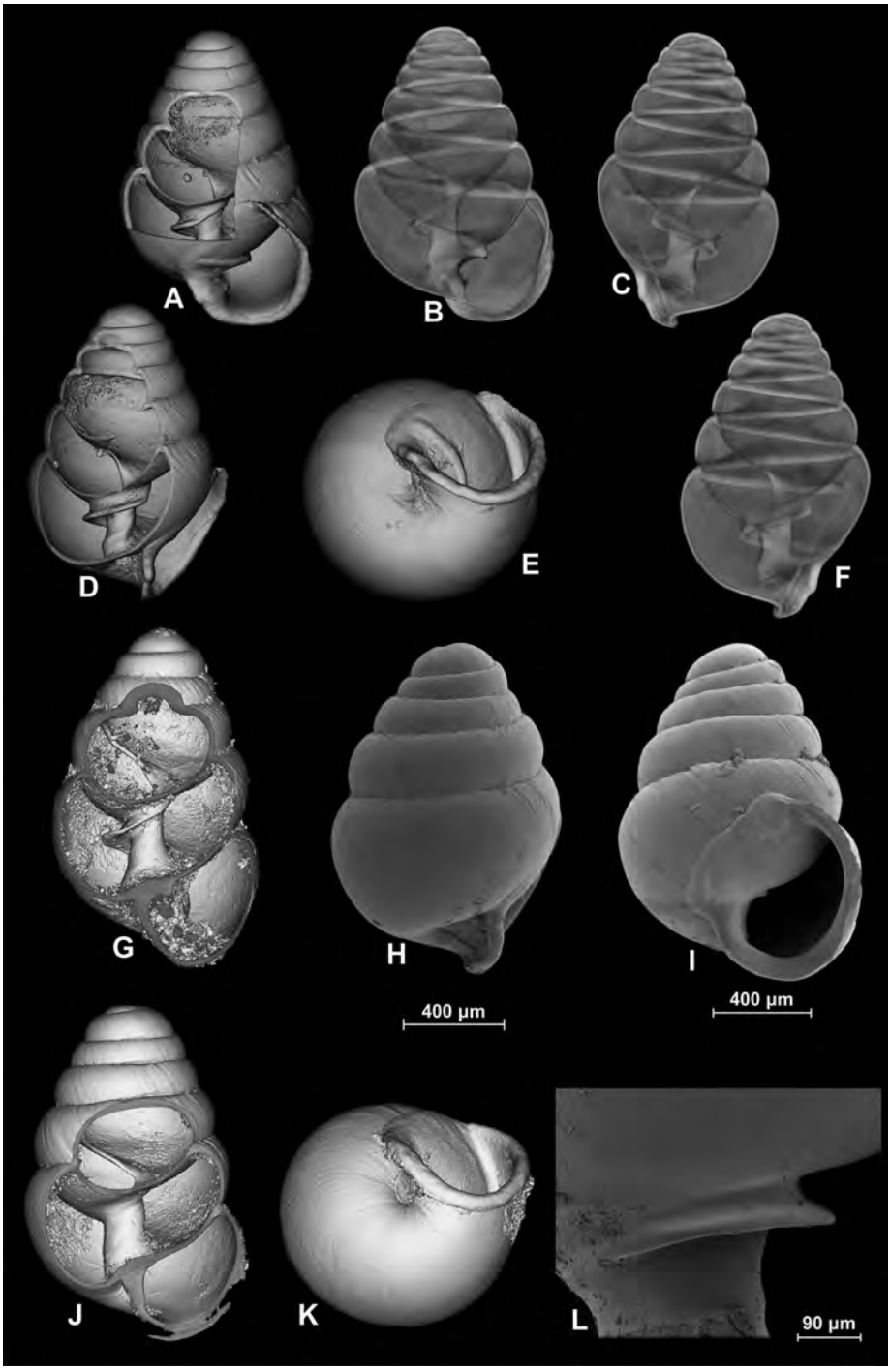

Figure 10. NanoCT–SEM Plate 4. **A–F** *Zospeum kupitzense* (RS3291), Loţekarjeva zijalka **G.** *Zospeum alpestre* (SMNH 2216), Kamniška jama **H, I–L** *Zospeum isselianum* Ihanščica cave **J–K** *Zospeum amoenum* (RS59), Potočka zijalka.

Figure 11. Type and shell images Plate 5: **A–E** *Zospeum kupitzense* holotype: (SMF 256354) **F–H** *Zospeum kupitzense* (SMNH 3291), Ložekarjeva zijalka **I–M** *Zospeum amoenum* (RS59), Potočka zijalka.

one season, and that these populations are/were not immediately threatened. Still, on a global scale, its distribution is fragmented and likely limited to far less than 312 caves within a radius of 20,000 km^2. In conjunction with the categories for the IUCN Red List (IUCN Standards and Petitions Subcommittee 2014), it can be considered a taxon of Least Concern (LC) since *Z. isselianum* is (potentially) known from many sites and these populations seem to be rather stable. Habitat disturbance through human agency poses the greatest threat.

Remarks. This species shows a wide range of variability in the shell. The parietal lamella with its nondescript shape and configuration has been considered characteristic

(Bole 1974, Slapnik 1991). The size and shape of the shell and aperture in conjunction with the visible parietal lamella indicate the very close relationship with *Z. alpestre* (see Bole 1974) and with *Z. amoenum* (see Slapnik 1991) in eastern Slovenia. The columella differs from congeners in this study by the presence of a secondary columellar dilatation at the base and an inclinate, extended columellar lamella. Superficial microstructure on the protoconch consists of spirally arranged pits interspersed by medium to wide zones of smooth non-pitted bands. The teleoconch shows a deep suture and irregular rows of tightly interconnected pits. Sometimes microscopic white chalky bands above and below the suture can be seen.

Consequence 2: Histological study of *Zospeum* sp. (Konečka zijalka) ultrastructure

Digestive system

As known for pulmonates, the anterior digestive system (Fig. 12) of *Zospeum* sp. (Konečka zijalka) has a muscular buccal mass containing the radular sac (rs) and a radula (r) bearing many teeth. The mouth is a slit located between the oral lappets. A crescent-shaped chitinous jaw (j) lies in the anterior part of the buccal mass (Fig. 12A). The jaw serves to press against and help grip the substratum, supporting interactions of the buccal mass during feeding (Morton 1955). In sync with the ellobioid scheme, the oesophagus (oe) is simplified (Barker 2001), bearing a thick cuticular sheath (cs) and extends straight into the unbranched section of the visceral cavity, where it widens into a thin-walled foregut. The anterior part of the visceral tract includes the oesophagus and foregut (fg), with associated, paired salivary glands (foregut glands) (sg) with ducts discharging into the posterior section of the buccal chamber (pharynx). The kidney (k) here is proportionately very large in respect to the overall size of this tiny snail. In our serial section (Fig. 12A) and in Dörge's (2010, unpublished data) partial 3D reconstruction (Fig. 15), the kidney encompasses ca. 14% of the entire volume of the cephalopedal mass. The heart complex (h) consisting of a single auricle and a single ventricle abuts the kidney at the bottom of the kidney sac. The foregut leads to the median section of the stomach, which is contained in a highly muscular girdle to form a contractile gizzard (g) (Fig. 16). A thick layer of cuticle (cu) lines the stomach. The muscular gizzard triturates the intesta (il), which in *Zospeum* sp. (Konečka zijalka) here, is composed of much mineral matter likely enriched with microorganisms and fungal tissue. The intesta of the stomach shows alkaline character inferable by the intense turquoise-coloured flecks of the toluidine stain. The elaborate muscular plates (mp) of the *Zospeum* sp. (Konečka zijalka) gizzard and the prevalent cuticle throughout the whole lumen (Fig. 16B) underscore the ellobioid tendency towards structural deviation of the gizzard as a grinding organ from the rest of the stomach (Barker 2001). The gizzard in *Zospeum* sp. (Konečka zijalka) here is overall much more elaborate than that of *Z. speleaum schmidtii* (Maier 1982, unpublished data). The thick epithelial wall of the stomach most in contact with the intesta shows columnar (cc) digestive cells

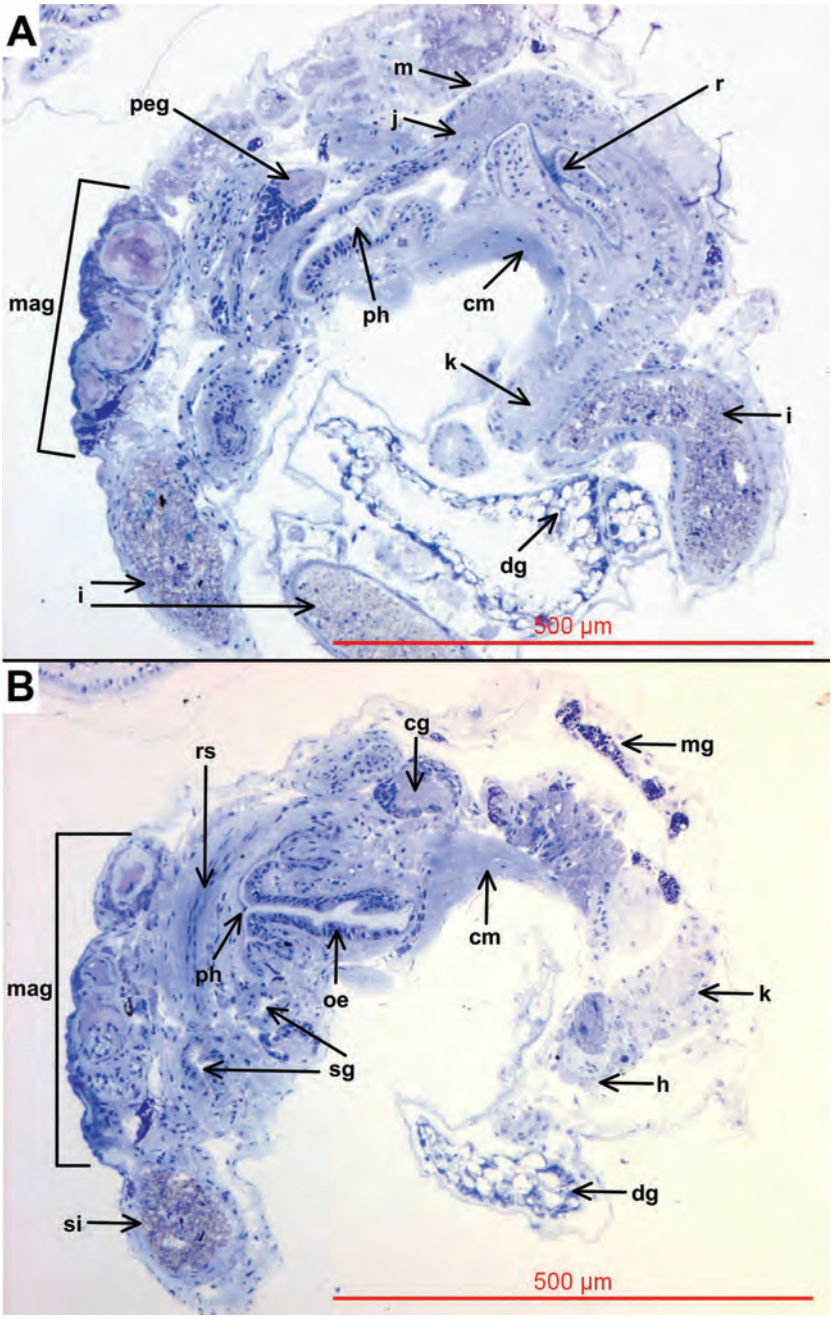

Figure 12. Light micrograph showing histological overview of the digestive system of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Mouth and buccal apparatus. Mouth slit between oral lappets (m), crescent-shaped jaw (j), radula (r), pharynx (ph), digestive gland (dg), intestine (i), kidney (k), columellar muscle (cm), and pedal ganglia (peg) **B** Mantle gland (mag), sigmoid intestine (si), radular sac (rs), cerebral ganglion (cg), mucus glands (mg), kidney (k), heart showing auricle and ventricle (h), digestive gland (dg), columellar muscle (cm), oesophagus (oe) and salivary glands (sg).

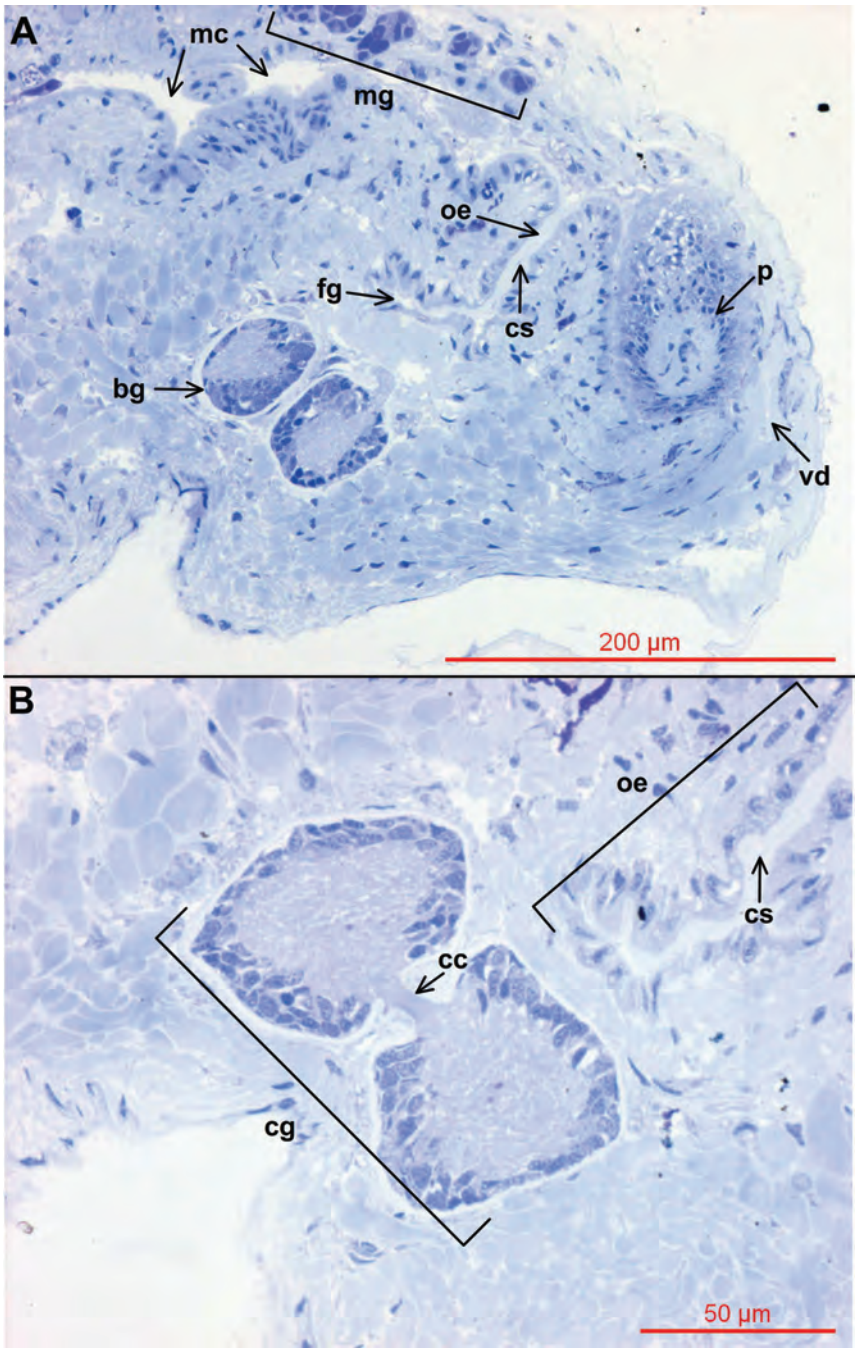

Figure 13. A Light micrograph showing the histological appearance of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675) with the mantel cavity (mc), mucus glands (mg), oesophagus (oe) bearing thick cuticular sheath (cs) and leading into the crop (c), the buccal ganglia (bg), and the cephalopodial portion of the reproductive system including penis (p) and vas deferens (vd) **B** Paired cerebral ganglia united by the cerebral commissure situated just dorsal to the origin of the oesophagus in the buccal mass.

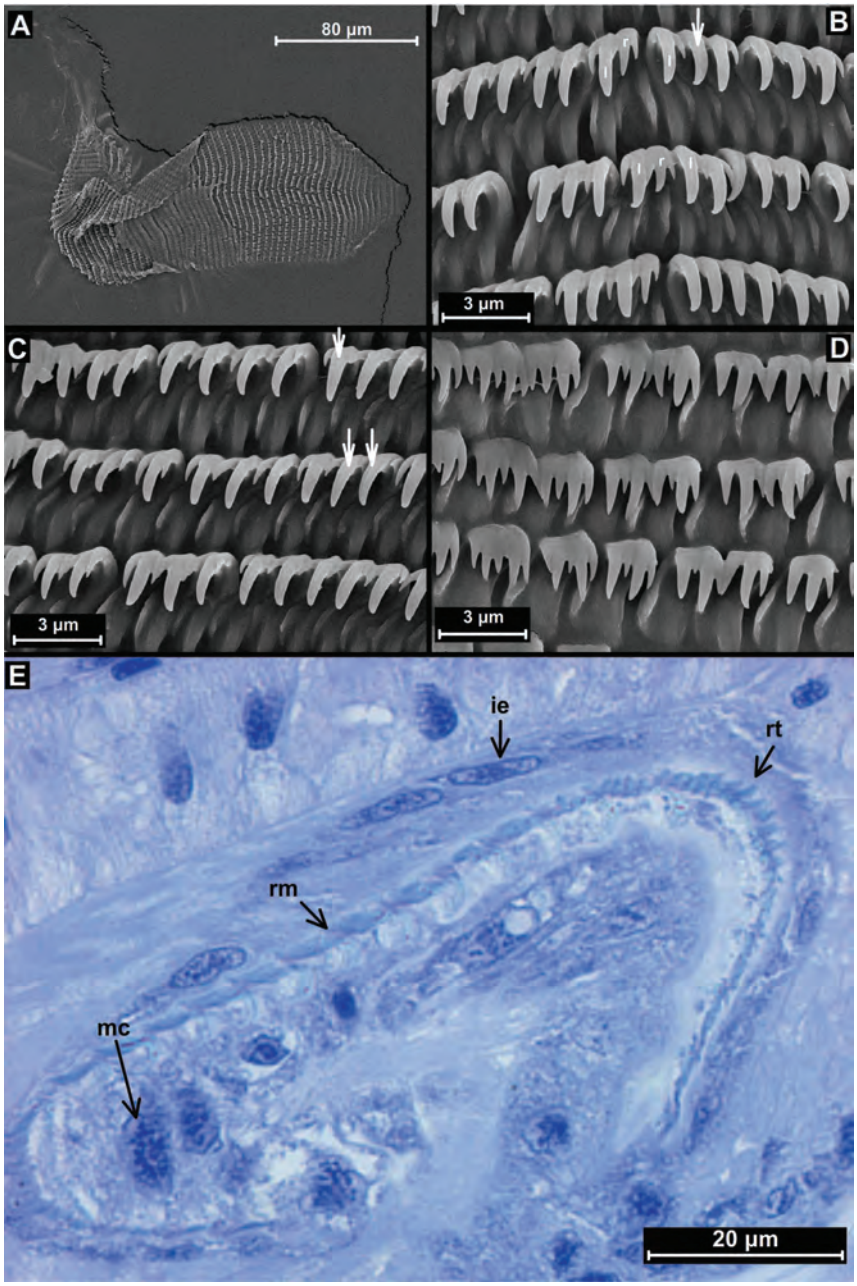

Figure 14. Images of the radula of *Zospeum isselianum* (Turjeva jama) (CSR SASA 37013 conspecific) and *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A–D** SEM of the radular ribbon of topotypic *Z. isselianum* **A** Radular ribbon showing adhesive layer of contact zone with odontophore **B** SEM overview showing rachidian teeth (r) and lateral teeth (l) **C** Rows of lateral teeth bearing fine medial groove **D** Marginal teeth **E** Light micrograph showing the histological appearance of the radular sheath of *Zospeum* sp. (Konečka zijalka) showing the inferior epithelium (ie) attached to the radular membrane (rm) mineralizing cells (mc) with degenerating nuclei and radular teeth (rt).

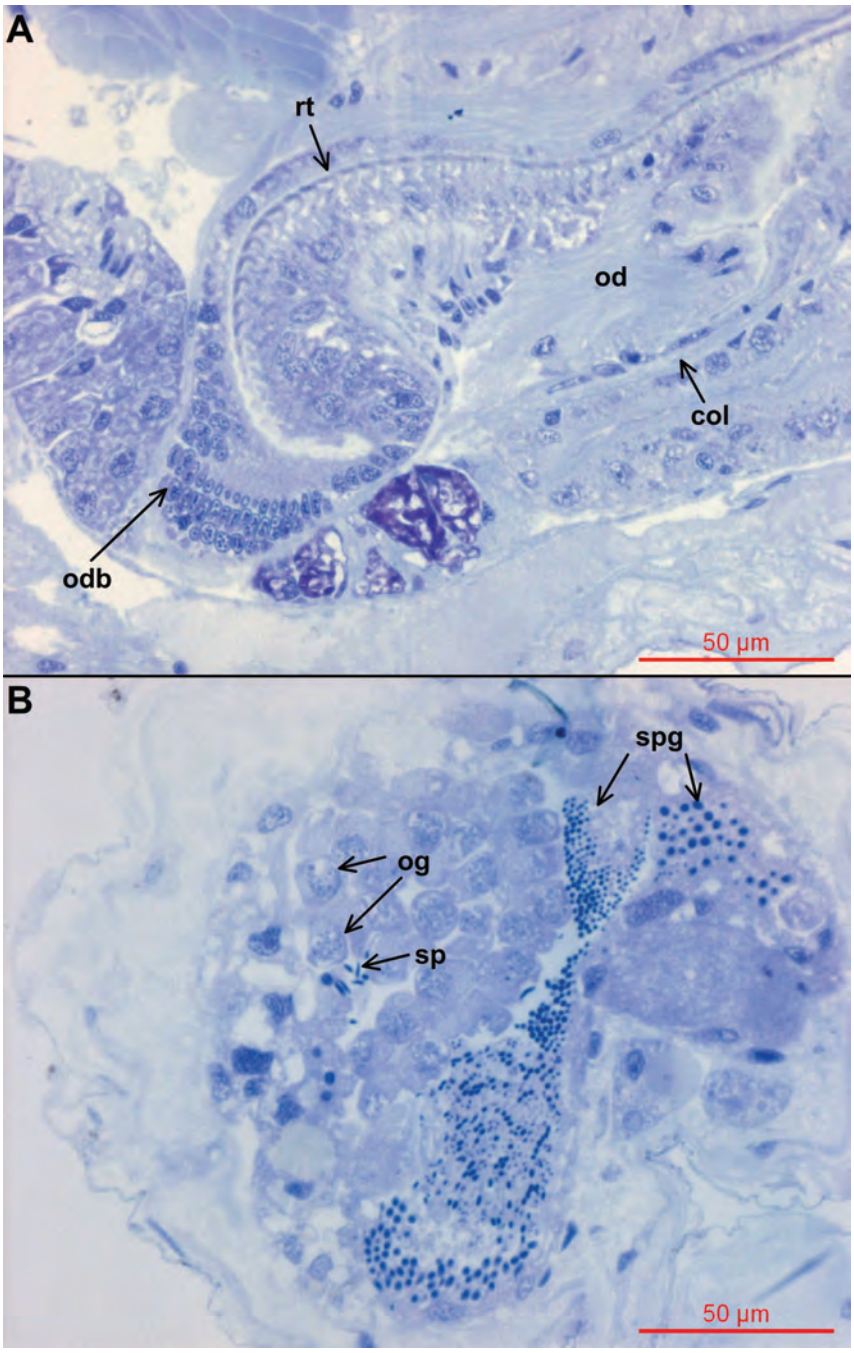

Figure 15. Light micrograph showing histological appearance of the radular complex of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Odontoblasts (odb) grouped in lower posterior section of radular sheath, radular teeth (rt), odontophore (od), and collostyle (col) **B** Section through an acinus of the ovotestis showing some stages of development of sustentacular cells (Sertoli cells) (sc) with spermatogonia (spg), spermatids (sp) and oogonia (og).

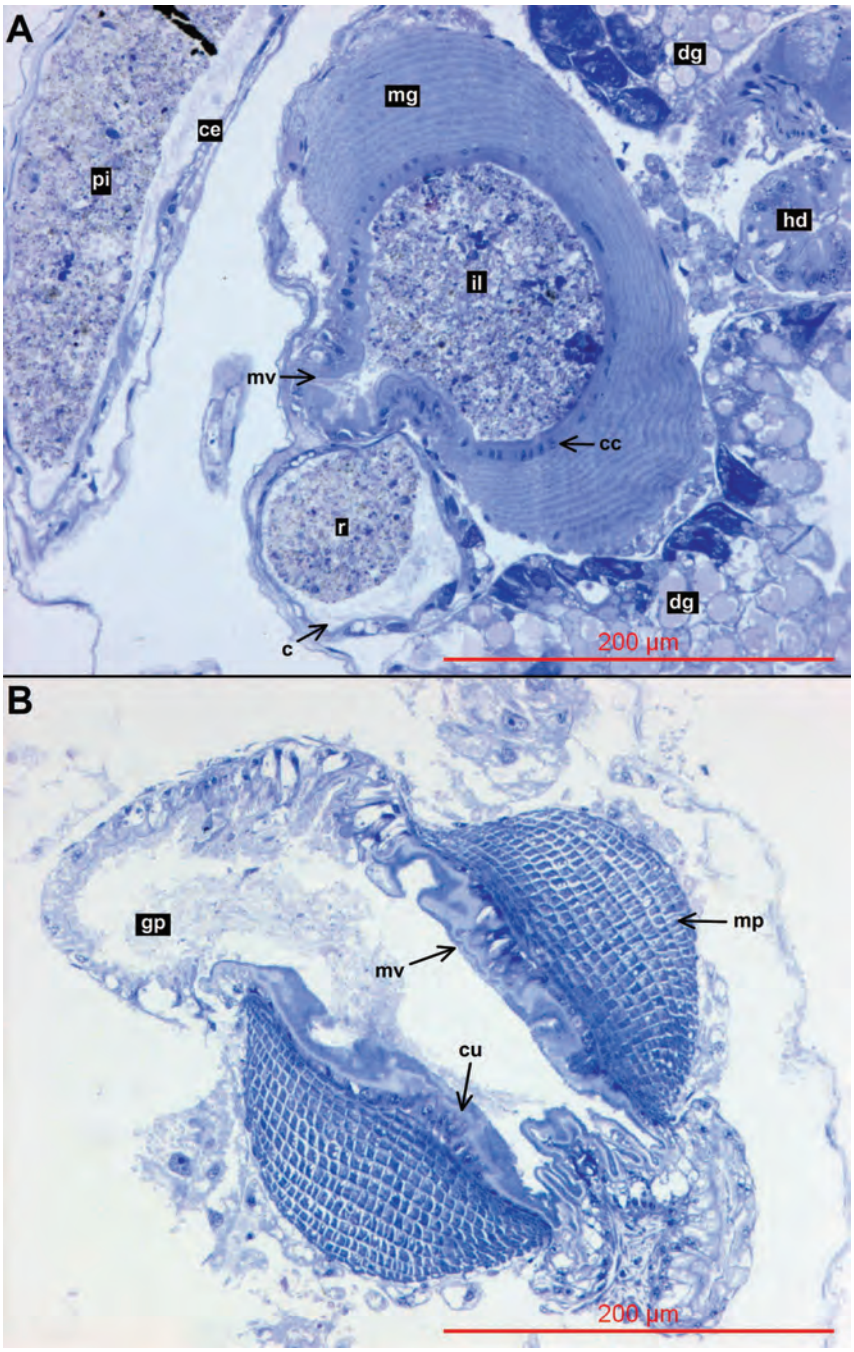

Figure 16. Light micrograph of the histological appearance of the digestive tract of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A–B** Bilobed muscular gizzard (mg) with epithelium showing columnar cells (cc) with microvillous apical surfaces (mv), posterior loop of the intestine (pi) with ciliated epithelium (ce) **B** Elaborate muscular plates (mp), thick cuticle (cu), highly ciliated rectum (r), gastric pouch (gp), digestive gland (dg) and hermaphroditic duct.

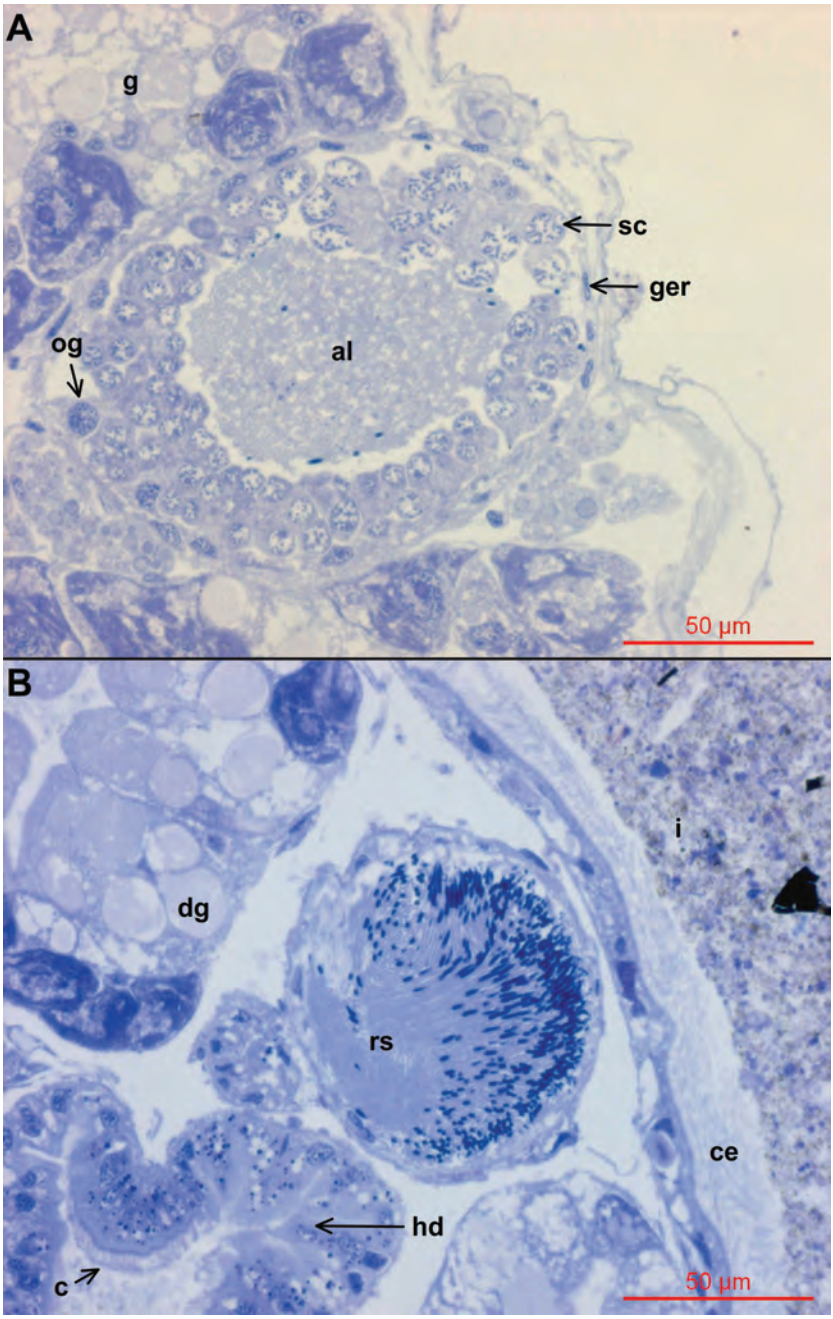

Figure 17. Light micrograph of a section of the reproductive tract of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Gonad (g) with acinus of the ovotestis including the germinal epithelial ring (ger) surrounding the acinar lumina (al), sustentacular cells (Sertoli cells) (sc) and oogonia (og) **B** Digestive gland (dg), hermaphroditic duct (hd) lined with cilia (c), posterior loop of the intestine (pi), receptaculum seminis (rs) containing allospermatozoa with spermatozoa oriented towards and apical ends touching the epithelial cells, and long motile cilia (ce) of intestine (i).

covered on the apical surface with microvilli (mv). The paired lobes of the glandular, voluminous digestive gland (dg) (Figs 12, 16, 17) are considered by Sumner (1965) to take on multiple functions in nutrient absorption, processing, secretion and excretion in pulmonate snails. In conjunction with the mantle gland (mg), the digestive gland is considered to provide materials for the organic crystalline matrix deposited in shell regeneration (Albolins-Krogis 1968).

The long intestine of *Zospeum* is visible from the outside of the shell when the snail is alive. The intestine encompasses an anteriorly directed loop frequently seen amidst other members of the Ellobioidea (Barker 2001) (Fig. 16). The posterior loop (pi) is large in surface area with an abundance of motile cilia (Fig. 16A). A remarkable degree of ciliation is also seen in the rectum. Long fecal strings are characteristic of the Carychiidae as well as for the Ellobioidea in general (Barker 2001).

Though not part of the digestive system but nonetheless encompassed in the broader scope of these slides, the mantle gland (mg) (Fig. 12) shows two different types of cellular composition, which more or less blend into each other within a circular, uniform layer of cuticle. This compact group of cellular packets is smaller and less extensive than that reported for *Z. spelaeum schmidtii* (Maier 1982, unpublished data).

Radula

The Carychiidae are considered to possess the simplest ellobioid radula (Martins 2007), bearing the typical symmetrical rachidian tooth accompanied by a series of lateral teeth and about the same number of tightly aligned marginal teeth. The radula of *Z. isselianum* (Turjeva jama, neotype locality) shows the tricuspate "*Carychium*-type" radula defined by Martins (2007) and reported by Jochum (2011, fig. 3) for *Zospeum* collected in Konečka zijalka. The cusps of the lateral and marginal teeth are flanked by an apposition of an additional denticle. The radular ribbon of topotypic *Z. isselianum* (Turjeva jama) shows the adhesive layer designating the zone of tensile strain and contact with the muscular odontophore (od) (Fig. 14A). In the histological section of *Zospeum* sp. (Konečka zijalka) (Fig. 15A), this subradular membrane is comprised of cuticula. It is formed at the opening of the radular sheath. The rachidian tooth (r) of *Z. isselianum* (Turjeva jama) is rather small (Fig. 14B). The mesocone is long while the ectocones are shorter and about one half the length of the mesocone. The tricuspate lateral teeth (l) are larger, bearing a slightly curved, uniformly long mesocone flanked by an endocone and an ectocone of differing lengths. The marginal teeth proceed from the laterals in cusp number from 3 to 4 to 5, whereby the third and fifth cusp of each tooth are reduced in length and the second and forth cusps are about the same length (Fig. 14D). In general, our observations match those of Giusti (1975, pl. 1) for *Z. tellenii* (=*Z. spelaeum costatum* (Freyer, 1855)) with the exception of a fine median groove midway down the mesocone in some of the lateral teeth (Fig. 14B–C). So far, this groove has only been observed on the more rounded, perhaps older teeth of the radulae from *Zospeum* sp. from Konečka zijalka (Jochum

2011, Dörge 2010, unpublished data) and from topotypic *Z. isselianum* here from Turjeva jama. As is known for pulmonates, the chitinized radular teeth are uniformly shaped in longitudinal columns, but vary markedly in the transverse direction on the radular membrane (Luchtel et al. 1997). The radular sheath is comprised of the radula membrane (rm), which is closely attached to the inferior epithelium (ie). Mineralizing cells (mc) can be seen in close contact with the developing teeth (Luchtel et al. 1997) (Fig. 14E).

Each column of teeth is formed by a single set of a number of odontoblasts (odb) located at the lower posterior end of the radular sheath as seen here for *Zospeum* sp. (Konečka zijalka) (Fig. 15A) (Mackenstedt and Märkel 1987). The shape, size and position of radular teeth are determined by the position, size and secretion rate of the odontoblasts (Mischor and Märkel 1984). These cells produce the matrix of the radula, whereas the superior and inferior epithelia (ie) (Fig. 14E) serve towards the maturation and transport of the *Zospeum* radula here (Mackenstedt and Märkel 1987). The colostyle (col) (Fig. 15A) is considered to play a role in supplying nutrients to the epithelia of the radular sac and may be integral in the disposal of degenerated cells (Mackenstedt and Märkel 2001).

Reproductive structures

One of the most prominent aspects of the cephalopedal region of *Zospeum* sp. (Konečka zijalka) here is the retracted conical penis and the long vas deferens embedded in the body wall, running parallel to but independent of the penis (Fig. 13A). The penial sheath in our cross section is sheer with this copulatory structure. The vas deferens follows a straight course before taking an abrupt left turn down the lower right hand side of the organ to meet the insertion point of the penis. This could possibly be due to contraction of the animal during preservation and not necessarily reflect the situation when the animal is alive.

The orange-yellowish gonad (when fresh) is situated between the uppermost lobes of the digestive gland in the hollow, apical whorls of the teleoconch (Fig. 8A–D). The lobes of the gonad comprise densely packed acini containing germinal cells (oogonia (og) and spermatogonia (sp)) and somatic Sertoli cells (supporting cells) (sc) and follicular cells (Fig. 17A). The male germ cells appear to develop and mature earlier in *Zospeum* sp. (Konečka zijalka) than the female germ cells (Fig. 15B). Diverse studies in other pulmonates have shown that oogenesis occurs in the acinar cortex and spermatogenesis in the compact, medullar region within the germinal ring (ger) situated in the neck of each acinus (Gómez 2001) (Fig. 17A). The acinar ducts of the ovotestis coalesce to form the hermaphroditic duct, whose projecting folds are lined by a dense layer of ciliated epithelium (Fig. 17B). The receptaculum seminis (rs) contains allospermatozoa, whose apical ends are oriented towards the epithelial wall (Fig. 17B).

Central nervous system (CNS)

The CNS of *Zospeum* sp. (Konečka zijalka) follows that of Maier (1982, unpublished data) described for *Z. spelaeum schmidtii* and Morton (1955), Martins (1996) and Harry (1998) for *Carychium*. Our observations conform, in part, to the general ellobioid plan of the central nervous system consisting of five pairs of ganglia (cerebral, pleural, pedal, parietal and buccal). However, since the position of the animal in the individual histological sections was constricted, the parietal and pleural ganglia could not be seen in this specimen. Martins (1996) described three types of CNS of which the Ellobiinian-carychiinian type (figs 24.1H–L) consists of a wide visceral ring with a very short parieto-visceral connective. In *Zospeum* sp. (Konečka zijalka), the two cerebral ganglia (cg) (Fig. 13B) are connected by an arching cerebral commissure (cc) over the oesophagus. In the immediate vicinity of the pharynx are the buccal ganglia (Fig. 13A). The pedal ganglion is particularly prominent due to its larger elongated form adjacent to the pharynx (Fig. 12A).

Consequence 3: Relegation of *Z. alpestre bolei* into synonymy of *Z. alpestre* with differential diagnosis of L3, including context to L2

Character analysis of the shell of allied species

Z. isselianum (RS0103) from Ihanščica cave (Figs 10H, I, L, 11I–M) though presented here in different perspectives, differs by the presence of a columellar lamella from the conchologically identified (RS) *Zospeum amoenum* from Potočka zijalka without a columellar lamella. This individual, though conchologically identified as *Z. amoenum* in a previous era, fits rather the morphology for *Zospeum* sp. as viewed from the backside of the ultimate whorl (Fig. 10H, I, L). Application of nano-CT enables a clear identification based on lamellar and columellar configuration of this shell. *Zospeum amoenum* from Potočka zijalka (Fig. 10J–K) on the other hand, has a smooth, non-elaborated cylindrical columella lacking a columellar lamella. In sync with morphological work by Bole (1974, fig. 3), the shell from Potočka zijalka is conchologically *Z. amoenum*.

The robust, broadly clavate columella of *Zospeum alpestre* of Kamniška jama (Fig. 10G) and the straight unelaborated cylindrical form of *Zospeum amoenum* from Potočka zijalka differ significantly from the slender, twisted forms of *Z. isselianum* from Turjeva jama (Fig. 8A–D) and *Z. isselianum* from Jama na Zgornjih Brsnikih (Fig. 8G–H). It appears that the smooth, robust columella in conjunction with the reduced, unelaborated columellar lamella of *Zospeum* sp. from Konečka zijalka (Fig. 8J) presents a unique, yet almost intermediary form to those of *Z. alpestre* (Kamniška jama) (Fig. 10G), *Z. amoenum* (Potočka zijalka) (Fig. 10J) and *Zospeum kupitzense* (Fig. 10A–F). The columellar lamella of *Z. alpestre* (Fig. 10G) on the other hand, is

thin and finely sinuous, extending to a brief horizontal plane within the penultimate whorl (Fig. 10G). The columellar lamella of *Zospeum* sp. (Konečka zijalka) (Fig. 8J) is robust and thick in contrast to the almost filigree form of the former. The lamella does not form a shelf-like projection but continues its slight, sinuous turn rather tightly about the columella, ending as a strong parietal element in the aperture.

Consequence 4: Differential diagnosis for *Z. kupitzense* (L4)

Zospeum kupitzense (Figs 9I–L, 10A–F, 11A–H) shows yet distinct and different shell morphology. Interconnecting shallow pits tend already on the protoconch to form differentiated concentric rows (Fig. 9–L). These rows develop into regularly spaced, incised orderly bands on the second whorl (Fig. 9J). By the third whorl, they become more densely spaced and irregularly wavy (Fig. 9L). Internally, *Z. kupitzense* shows a rather slender but more truncated columella than in *Z. isselianum* (Turjeva jama) (Fig. 8A–D) and *Z. isselianum* from Jama na Zgornjih Brsnikih (Fig. 8G–H). However, like the two *Z. isselianum* forms, *Z. kupitzense* shows a secondary columellar dilatation at the base of the columella, which forms the shallow, but more obliquely positioned umbilical notch. The columellar lamella (Fig. 10A–D, F) maintains a similar, slight sinuous extension in sync with the contour of that in *Z. alpestre* (Fig. 10G). However, the columellar lamella does not configure into a shelf-like extension but rather, maintains a well-confirmed tight course around the columella, carrying the pronounced parietal continuation of this course all the way to the outermost extension of the aperture (Fig. 10E). This robust continuation of the columellar lamella forms a thick ridge extending as far as and aligning directly above the umbilical notch of this species (when viewed from below). This external feature clearly distinguishes *Z. kupitzense* from the others.

The umbilical notch of *Z. isselianum* (Turjeva jama, neotype locality) (Fig. 8E) is less emphasized in the appearance of forceful twisting as in *Z. kupitzense*. This emphasized twisting dynamic is evident in the nano-CT images of *Z. kupitzense* (Fig. 10A–F). The umbilical perspective of *Zospeum* sp. (Konečka zijalka) (Fig. 8I–L) shows a deeply set umbilicus for this morphospecies. Although taken from a fragment of the umbilical region (Fig. 8I), this view is informative enough to indicate that this notch is deeper set than in the other allied species comprising this study. The columellar lamella maintains a similar, slight sinuous extension as in *Z. alpestre* (Fig. 10G).

The deep interconnected pits forming continuous rows of wavy pattern on the upper teleoconch are markedly more pronounced in topotypic *Z. isselianum* (Turjeva jama) (Fig. 9A–F) than in *Zospeum* sp. (Konečka zijalka) (Fig. 9G–H). The interconnected shallow pitted rows on the teleoconch of *Z. amoenum* (Fig. 9M) are less pronounced and rather intermediary and moderate compared to the clear and well-defined topography of the rows in topotypic *Z. isselianum* (Turjeva jama) (Fig. 9A–F) and the deeply pitted waves of *Z. kupitzense* (Fig. 9I–L). However, the pitting on the protoconch of *Z. amoenum* (Ihanščica cave) is well pronounced though not distinctive to this species. Although pitting was well defined on the protoconch of all species in

this study, *Z. isselianum* (Turjeva jama) shows a distinctive pattern of densely set pits interspersed by bands of empty space and then more rows (Fig. 9D–E). Rheomorphic stretching is frequently encountered on the protoconch of *Z. isselianum* (Fig. 9A, D).

Discussion

Our molecular delimitation results singularly imply that:

a) *Zospeum isselianum* (L1) is validated by molecular data from the area of the new precise type locality (Turjeva jama, Robič, Kobarid, Slovenia).
b) *Zospeum* sp. (Konečka zijalka) (L2) is an unknown, highly morphologically variable *Zospeum* lineage (comprising specimens of *Z. isselianum* and *Z.* cf. *amoenum*) with ambiguous identity.
c) Polytypy within the species *Z. alpestre* (i.e. Z. *alpestre alpestre*, *Zospeum alpestre kupitzense* and *Zospeum alpestre bolei*) needs reassessment.

Our studies of *Zospeum* shell morphology of the allied Eastern Alpine species show the wide degree of external shell variability and evidence of convergent evolution within these taxa. These investigations corroborate the findings of Bole (1974), Maier (1982, unpublished data), Slapnik (1991) and De Mattia (2003) in that *Zospeum* shells typically tend to be very variable in shape from one population to the other and frequently show wide variability within the same population. The most reliable conchological character, however, in differentiating between morphospecies in this study is the form and configuration of the columella. The columellar lamella on the other hand, is subject more to phenotypic variability in the degree of shell material invested in the composition of the lamella and the subsequent expression of planar extension. The external superficial texture of the shell was found to be differentiable in *Z. isselianum* and *Z. kupitzense* in this study whereby the pattern of microstructural pits on the shells of *Z. isselianum* and *Z. kupitzense* are specific for these two species.

The age of the shell, superficial encrustations, degree of preservation/freshness, and the degree of biomineralization affecting shell hardness vary, and thus, generally influenced moderate forms of superficial shell texture.

Zospeum shows characteristic pulmonate design with ellobioid affinity. The gross anatomy of *Zospeum* (Konečka zijalka) does not deviate significantly from other investigated *Zospeum* species (Giusti 1975, Maier 1982, unpublished data, Dörge 2010, unpublished data). The *Zospeum* individual in Dörge's (2010, unpublished data) cursory histological study was collected at the same time and came from the same population as the specimen presented here. Some structures such as statocysts in conjunction with the pedal ganglion were recorded as was a large albumen gland, suggesting that Dörge's (2010, unpublished data) specimen was likely collected in the female phase. In the Ellobioidea, the penial structure shows varying degrees of complexity (Martins 1998). In anatomical studies conducted on *Zospeum* so far, individuals in the male phase predomi-

nated (Giusti 1975, Maier 1982). The albumen gland was not detected in our study and may not yet have developed or may have been reduced on account of the male phase. The individual used in this study shows a conical, well-retracted penis into the body cavity, indicating the animal was collected during the male phase but not sexually active (Duncan 1975, Gómez 2001). As in most ellobioid genera, excepting for *Blauneria* Shuttleworth (Ellobiinae) and *Myosotella* Montserato (Pythiinae) (Barker 2001), the long vas deferens of *Zospeum* is embedded in the body wall and courses parallel to, but, independent of the penis. Maier's (1982, unpublished data) investigation of *Z. spelaeum schmidtii* shows a likely more mature state in size and elongated form as well as an apparent separation of the penial sheath from the penis versus the compact form and sheerness of the penial sheath in *Zospeum* (Konečka zijalka) here. The specimen in Maier's study was likely sexually active. In *Z. tellinii* (=*Z. spelaeum costatum* (Freyer, 1855)), Giusti (1975) described the penis as being extended and long as well as positioned outside the body parallel to the right tentacle. Our specimen (and that of Dörge (2010, unpublished data)) was collected in August and likely reflects seasonal protandry in *Zospeum*.

The radulae of *Zospeum isselianum* (Turjeva jama) here and *Zospeum* sp. (Konečka zijalka) (Dörge 2010, unpublished data; Jochum 2011) show a long median groove on some of the lateral teeth, which is a new structural feature for the "*Carychium*-type" radula defined by Martins (2007) for the Carychiidae. Diverse considerations along a second line of this study involve ecological aspects such as the relationship between tooth cusps and substrates, producing adaptive moderations of morphological detail. Mechanical factors such as forces acting on teeth during feeding produce adaptive solutions to pressure, such as development of compressional ridges (Hickman 1980). Moreover, the cusp of the tooth interacts with the substrate during feeding and is vulnerable to substrate characteristics and dietary preferences (Hickman 1980; Luchtel et al. 1997). Consequently, if tooth cusps are to most effectively function with the fine nuances of substrate composition and configuration (i.e. "grain"), superimposed elaborations likely evolve to correlate with the substrate grain. The grooves on the radulae of these two *Zospeum* here may well reflect such ecologically induced adaptive factors.

Based on the most unambiguous shell character found in this study, namely, the configuration of the columella and independently, the relationship of the columellar lamella with it, the bigger morphological assessment-vs-molecular delimitation picture can be broken down as follows:

- Columella slender, twisted; secondary columellar dilatation at base of columella; columellar lamella inclinate, extended. Lineage 1: *Zospeum isselianum* from new type locality.
- Columella robust, cylindrical, smooth; no columellar lamella: *Z. amoenum* (Potočka zijalka).
- Columella robust, hint of secondary dilation at base, smooth; reduced, un-elaborated (i.e. non-extended) columellar lamella. Lineage 2: *Zospeum* sp. (Konečka zijalka) appears to be morphologically an intermediary form of *Z. amoenum* and *Z. isselianum* morphotypes.

- Columella broadly clavate; columellar lamella thin and finely sinuous, extending to a short horizontal plane within the penultimate whorl. Lineage 3: *Z. alpestre* (Kamniška jama).
- Columella slender, twisted; slight secondary columellar dilation at base of columella; columellar lamella only similar to *Z. alpestre* but not the columella; pronounced parietal lamella formed from the inner track of the columellar lamella; parietal lamella remarkably long, extending to middle of shell above the umbilicus (bottom perspective). Lineage 4: *Z. kupitzense*.

This morphological analysis, in conjunction with the molecular results, suggests that lineage 2 *Zospeum* sp. (Konečka zijalka) possesses an intermediary columellar form and shows morphological affinity to both *Z. amoenum* and *Z. isselianum*. We cannot designate species status for this material at this time. On the one hand, these individuals are not *Z. isselianum* and can thus, be excluded as this species by our study here. On the other hand, *Z. amoenum* cannot be excluded because we did not investigate type-material of this species. This means the examination of L2 should be the focus of another investigation, incorporating topotypic material of *Z. amoenum* (locus typicus: Velika Pasjica, Gornji Ig, Slovenia) to clarify this issue. Interestingly, this cave is also the type locality of *Zospeum spelaeum schmidtii*. In regards to the bigger picture here, it is remarkable that Bole (1974) recorded three species of *Zospeum* from Ihanščica cave (*Z. alpestre isselianum*, *Z. spelaeum schmidtii* and *Z. amoenum*) while the Kuščer collection in Trieste houses another specimen of *Z. spelaeum* (Rossmässler, 1839) (Kuščer collection nr. 1186) from this very cave (figs 19–21, De Mattia 2005). In addition, this same cave was the origin of a specimen identified by Kuščer (*Z. schmidti striatum* nov. sp, NHMW Edlauer #48.657), which is currently recognized as *Z. spelaeum schmidtii*. In our study, specimens of *Z.* cf. *amoenum* (Fig. 9M–O) and *Z. isselianum* (Fig. 10H,I,L) also came from this cave. In context, we are likely witnessing Ihanščica cave as the site of a "species flock" whereby this monophyletic, relatively species-rich (for *Zospeum* at least), narrowly endemic group of closely related taxa all inhabit the same subterranean ecosystem (Bickford et al. 2006). On the other hand, Ihanščica cave and Konečka zijalka are ca. 40 km apart, noncontiguous and share no common river drainage systems where the *Z. isselianum* populations comprising this study could come in contact. Further investigation of *Zospeum* sp. (Konečka zijalka) may likely show a similarly complicated dynamic involving a very high regional/within-cave morphological variability producing many distinct morphotypes. Thus, the characterisation of several *Zospeum* morphospecies within Ihanščica cave could also be seen as a taxonomic over-interpretation of the intraspecific variability of a single species or a few species.

Based on the configuration of the columella plus the independent configuration of the columellar lamella, morphological differentiation of these lineages was generally possible. Although aperture dimensions and the expression of the columellar lamella in the aperture were highly rated in earlier assessments of *Zospeum* (Bole 1974), the value of these characters has been exaggerated. Since the columella functions as a form of scaffolding for the shell, it must conform to the internal needs and static requirements

of the animal. The size of the aperture is more a reflection of the size of the foot. More important however, is the mechanization of the columellar muscle during mobility, during the protraction of the penial organ and the role of the columellar muscle in radular function as these aspects largely have much to do with the configuration of the columella and the utilization of the columellar lamella for these specific processes.

Conclusions

Four evolutionary lineages of Eastern Alpine *Zospeum* were clearly molecularly delineated and assessed using various non-molecular methods. Based on differentiating shell features and the molecular species delimitation, a neotype for *Zospeum isselianum* was described. This study performed the first, non-destructive 3D-reconstruction using X-ray tomography (nano-CT) to differentiate shells of conchologically and geographically allied species of *Zospeum*. Since external shell morphology is too variable, specific focus was given to internal characters such as to the configuration of the columella and the orientation of the columellar lamella within the ovate conical shell. The columella was found to be the most reliable conchological character differentiating species of Eastern Alpine *Zospeum* taxa. SEM assessment infers that a median groove incised in the middle cusp of the lateral teeth of *Z. isselianum* (Turjeva jama, neotype locality) here and *Zospeum* sp. (Konečka zijalka) of previous investigations (Dörge 2010, unpublished data, Jochum 2011) reflects a phenotypic modification in intrinsic design. Seasonal protandry is inferred for *Zospeum*. New access to new means of interpretation has provided a refreshing taxonomic perspective for these microscopic ellobioid snails. Considering the results of Weigand et al. (2013) and our conchological investigations here, we find no reason to maintain the two subspecies entities currently comprising the *Z. alpestre* complex of the Eastern Alpine region. This study reduces the volume of previous *Z. alpestre*-laden eras by narrowing the status to two valid species: *Z. kupitzense* and *Z. alpestre* for this region. On the other hand, *Zospeum* sp. (Konečka zijalka) (L2) presents a new dilemma we didn't have before, namely, an intermediate form of *Z.* cf. *amoenum* and *Z. isselianum* in an morphologically amalgamated, *Z. amoenum–Z. isselianum* lineage. This incidence of character amalgamation likely reflects a condition involving a species flock and potential incipient speciation here. However, other plausible causes could involve hybridization or the use of a single gene fragment for delimiting species. Future investigations should focus on clarification of *Zospeum* sp. (Konečka zijalka) by including material of *Zospeum amoenum* collected at its type locality (Velika Pasjica, Gornji Ig, Slovenia). Our study lays the groundwork for future taxonomic investigations of *Zospeum*. We emphasize the value of applying a multifaceted, taxonomic approach to assess the frequent instances of intraspecific variability in other groups of highly morphologically variable species and not only for Southeastern Alpine *Zospeum* taxa (Schniebs et al. 2011, Puzin et al. 2014). Lastly, we hope this investigation presents valuable insights for future biodiversity studies of subterranean taxa as well as bears potential for influencing conservation management strategies within the Southern Alpine subterranean realm.

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Supplementary material 1

List of localities of *Z. isselianum*

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Data type: occurrence

Explanation note: List of localities of *Z. isselianum*. A – Austria, BiH – Bosnia and Herzegovina, Cro – Croatia, Slo – Slovenia, Ita – Italy, CBSS – Collection of Croatian Biospeleological Society, CSR SASA – Malacological collection of the Biological Institute of the Centre for Scientific Research of the Slovenian Academy of Sciences and Arts in Ljubljana, SMNH – Malacological collection of the Slovenian Museum of Natural History, LIT – data from literature.